DEVELOPMENT AND APPLICATION OF A HIGH-RESOLUTION FLOW INJECTION ANALYSIS FOURIER TRANSFORM MASS SPECTROMETRY (FIA-FTMS) METHOD TO IDENTIFY AND QUANTIFY LIPIDS IN HUMAN FECAL SAMPLES

Dissertation
zur Erlangung des Doktorgrades
der Biomedizinischen Wissenschaften
(Dr. rer. physiol.)

der
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der Universität Regensburg

vorgelegt von
Verena Ertl
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II List of Abbreviations

3PLE	Three-Phase Extraction
5-HETE	5-Hydroxyeicosatetraenoic Acid
ALA	n-3-α-Linoleic Acid
AGC	Automated Gain Control
B&D	Bligh and Dyer
BUME	Butanol/Methanol Extraction
CE	Cholesteryl Ester
Cer	Ceramide
CID	Collision Induced Dissociation
CL	Cardiolipin
CV	Coefficient of Variation
DB	Double Bond
DC	Direct Current
DG	Diacylglycerol
DIMS	Direct Infusion Mass Spectrometry
DMAPP	Dimethylallyl Pyrophosphate
dw	Dry Weight
EECs	Enteroendocrine Cells
ESI	Electro-Spray-Ionization
FA	Fatty Acyl
FAME	Fatty Acid Methyl Ester
FC	Free Cholesterol
FIA	Flow-Injection Analysis
FTMS	Fourier Transform Mass Spectrometry
FWHM	Full Width at Half Maximum
GABA	γ-Aminobutyric Acid
GC-MS	Gas Chromatography Mass Spectrometry
GI	Gastrointestinal
GL	Glycerolipids
GluCer	Glucosylceramide
GP	Glycerophospholipids
HDC	Higher-Energy Collisional Dissociation
HexCer	Hexosylceramide

HILIC	Hydrophilic Interaction Liquid Chromatography
HR	High Resolution
ICR	Ion Cyclotron Resonance
IS	Internal Standard
IT	Injection Time
IPP	Isopentenyl Diphosphate
K2CO3	Potassium Carbonate
LA	n-6 Linoleic Acid
LCF	Lipid Class Selective Fragment
LCFA	Long-Chain Fatty Acids
LC-MS	Liquid Chromatography
LIT	Linear Quadrupole Ion Trap
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
LPA	Lysophosphatidic Acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
MA	Methylamine
MALDI	Matrix-Assisted Laser Desorption
MCFA	Medium-Chain Fatty Acid
MG	Monoacylglycerol
MLF	Molecular Lipid Species Specific Fragment
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MTBE	Methyl-Tert-Butyl Ether
MUFA	Monounsaturated Fatty Acid
MW	Molecular Weight
m/z	Mass-to-Charge Ratio
NEFA	Non-Esterified Fatty Acyl
NL	Neutral Loss
NMR	Nuclear Magnetic Resonance
PA	Phosphatidic Acid

PBS	Phosphate Buffer Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PK	Polyketides
PR	Prenol Lipids
PS	Phosphatidylserine
PRM	Parallel Reaction Monitoring
PUFA	Polyunsaturated Fatty Acid
QqQ	Triple Quadrupole Mass Analyzer
RF	Radio Frequency
RT	Room Temperature
S1P	Sphingosine-1-Phosphate
SCFA	Short-Chain Fatty Acid
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SL	Saccharolipids
SM	Sphingomyelin
sn	Stereospecific Numbering
SP	Sphingolipids
SPE	Solid-Phase Extraction
SRM	Selected Reaction Monitoring
ST	Sterol Lipids
TG	Triacylglycerol
TMA	Trimethylamine
TOF	Time-of-Flight

III Zusammenfassung

Für die menschliche Gesundheit sowie auch für Krankheiten spielt das Darmmikrobiom eine wichtige Rolle. Fäkalien spiegeln dabei die mikrobielle Aktivität wider. Die Analyse der fäkalen Metaboliten liefert daher Einblicke in die metabolischen Wechselwirkungen zwischen Darmmikrobiota und Wirtsorganismus.

In dieser Arbeit wird die Entwicklung einer Fließ-Injektion-Analyse beschrieben, welche mit einer Fourier-Transform-Massenspektrometrie-Messung (FIA-FTMS) gekoppelt ist, um Lipidspezies in menschlichen Fäkalien mit einem hohen Probendurchsatz zu identifizieren und quantifizieren. Fäkale Homogenate wurden gemäß dem Protokoll von Bligh und Dyer einer Lipidextraktion unterzogen und mittels FIA-FTMS analysiert. Die Methode wurde an einer Q Exactive hybrid Orbitrap mit einer maximalen Auflösung von 140,000 bei *m/z* 200 entwickelt. Kurze Messzeiten von weniger als vier Minuten und eine automatisierte Datenauswertung unter Verwendung der ALEX-Software und selbstprogrammierter Makros in Microsoft Excel ermöglichten einen hohen Probendurchsatz.

Die Analyse von Fäkalien verschiedener Probanden ergab eine große Heterogenität der Lipidkonzentrationen. In der Mehrzahl der Proben wurden Triacylglycerin- (TG) und Diacylglycerin-Spezies (DG) detektiert, welche durch MS2-Spektren verifiziert werden konnten. Daher konzentrierte sich die Quantifizierung hauptsächlich auf diese beiden Lipidklassen. Die Methodenvalidierung umfasste Experimente zur Nachweisgrenze, Linearität, Bewertung von Matrixeffekten, Wiederfindung und Reproduzierbarkeit. Die durchgeführten Validierungsexperimente zeigten eine gute Reproduzierbarkeit, mit Ausnahme von etwa 10% der Proben, bei welchen ein CV von mehr als 15% beobachtet wurde. Die beeinträchtigte Reproduzierbarkeit war auf die Probeninhomogenität zurückzuführen und konnte auch durch zusätzliche Probenvorbereitungsschritte nicht verbessert werden. Zudem zeigten diese Experimente höhere Mengen an DG-Spezies, wenn die Proben in 2-Propanol statt in wässriger Lösung homogenisiert wurden, was sich vermutlich auf die Lyse von Bakterien und eine erhöhte TG-Lipolyse zurückführen lässt. Diese Effekte waren probenspezifisch und untermauerten die hohe Heterogenität der fäkalen Materialien sowie die Notwendigkeit einer weiteren Evaluierung der präanalytischen Bedingungen.

Eine Messung im negativen Ionenmodus durch Zugabe von Methylamin ergab nur sehr niedrige Signale für *Lyso*-Phospholipide und Glycerophospholipide, welche nicht verifiziert werden konnten. Hieraus lässt sich schließen, dass für die Analyse dieser Lipidklassen empfindlichere Methoden wie z.B. LC-MS benötigt werden.

Zusammenfassend konnte gezeigt werden, dass FIA-FTMS ein schnelles und genaues Verfahren zur Quantifizierung von DG- und TG-Spezies darstellt, welches geeignet ist, einen Einblick in das fäkale Lipidom zu geben und dessen Rolle in Gesundheit und Krankheit zu entschlüsseln.

IV Abstract

The intestinal microbiome plays an important role in human health and disease and fecal materials reflect the microbial activity. Thus, analysis of fecal metabolites provides insight into metabolic interactions between gut microbiota and host organism. In this work, we applied flow injection analysis coupled to Fourier transform mass spectrometry (FIA-FTMS) to identify and quantify lipid species in human fecal samples in high throughput. Fecal homogenates were subjected to lipid extraction according to the protocol by Bligh and Dyer, and analyzed by FIA-FTMS. The method was developed using a Q Exactive hybrid Orbitrap with a maximum resolution of 140,000 at *m/z* 200, a short analysis time of less than four minutes and an automated data evaluation using the ALEX software and self-programmed macros in Microsoft Excel.

The analysis of different subjects revealed a vast heterogeneity of lipid species abundance. The majority of samples displayed prominent signals of triacylglycerol (TG) and diacylglycerol (DG) species that could be verified by MS2 spectra. Therefore, we focused on the quantification of TG and DG. Method validation included limit of quantification, linearity, evaluation of matrix effects, recovery, and reproducibility. The validation experiments demonstrated the suitability of the method, with exception of approximately 10% of samples in which we observed CVs higher than 15%. Impaired reproducibility was related to sample inhomogeneity and could not be improved by additional sample preparation steps. Additionally, these experiments demonstrated that, compared to aqueous specimens, samples containing isopropanol showed higher amounts of DG, presumably due to lysis of bacteria and increased TG lipolysis. These effects were sample-specific and substantiate the high heterogeneity of fecal materials as well as the need for further evaluation of pre-analytic conditions.
Despite optimization of ionisation by addition of methylamine FIA-FTMS of fecal lipid extracts in negative ion mode revealed low signals for *lyso*-phospholipids and glycerophospholipids that could not be verified. This suggests that analysis of these lipid classes requires more sensitive methods like LC-MS.

In summary, FIA-FTMS offers a fast and accurate tool to quantify DG and TG species and is suitable to provide insight into the fecal lipidome and its role in health and disease.

1. Introduction

Lipids are an important class of biomolecules in living organisms. Due to their hydrophobic nature, lipids provide the ability to separate living entities from their natural surroundings. They originate entirely or in part from carbocation-based condensations of isoprene units and/or from carbanion-based condensations of thioesters (1). The lipid species possess different aliphatic chains and various polar head groups which are differentially connected to the head groups. The aliphatic chains of lipids vary in the number of carbon atoms, degree of unsaturation, location of double bonds, and potential branches (2). Besides the important function of energy storage, lipids serve as building blocks in cellular and subcellular membranes, and as signaling molecules (3). Cellular lipids are very dynamic and highly complex. At the level of attomole to nanomole of lipids per mg of protein tens to hundreds of thousand possible molecular lipid species could be present in the cellular lipidome (4). Therefore, the reliable and accurate quantification of lipid species is important (5). Since lipids play a crucial role in many biological processes, any imbalance in sensing and signaling pathways can cause cardiovascular disease, neurodegenerative diseases, and type 2 diabetes mellitus by promoting atherosclerosis and chronic inflammation (6, 7). Additionally, lipids are involved in basic processes essential for tumor development, for example, cell growth, proliferation, differentiation, and motility (8).

Lipids play a role in the last step of the “omic” cascade in which metabolome is gradually developed from genome via transcriptome and proteome (9). Lipidomics, a subgroup of metabolomics, is a relatively new disciplinary research field due to the inherent chemical complexity of the lipidome and the consequent challenges associated with analysing it (10). Within the last decade, the number of publications has increased rapidly, by a factor of 7.7 according to Web of Science, which makes it one of the fastest growing research fields (10). Lipidomics has recently emerged because of rapid advances in developing new mass spectrometric protocols and techniques due to its sensitivity and specificity (11). Therefore, dynamic changes of lipids during physiological or pathological processes can be determined by analyzing lipid structures, mass levels, cell functions, and interactions in a spatial and temporal fashion (2). Over the last decades, several mass spectrometric lipidomic methods with clinical and scientific application have been adapted (12-14).

1.1 Lipid Categories

Lipids, a heterogeneous pool of compounds, contain either fatty acyl/alkyl, sphingosine, or isoprene moieties as their hydrophobic building block. Lipid Maps consortium has classified lipids into eight categories in 2005. Based on the publication of Fahy et al. (1), lipid species are divided into following categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK). Sterol and prenol lipids are derived from the condensation of isoprene subunits, whereas other lipid classes are synthesized from ketoacyl subunits (15). Each of the eight lipid categories comprises further lipid classes and subclasses.

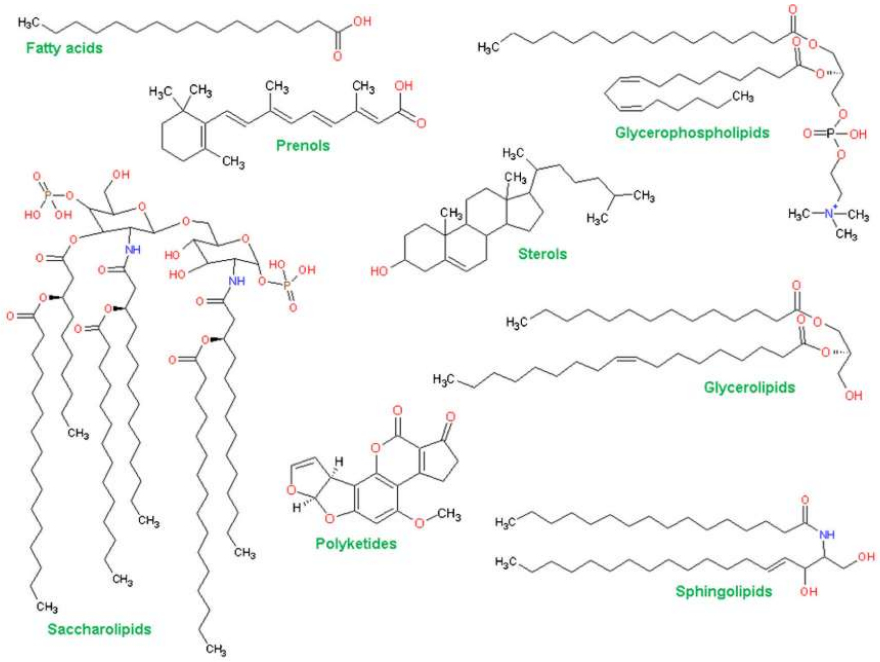


Figure 1.1: Examples of the eight lipid categories according to the International Lipids Classification and Nomenclature Committee. Reprinted with permission from Springer Nature and Creative Common Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Copyright Züllig, T.; Trötzmüller, M.; Köfeler, H.; 2020 (16).

This chapter will briefly introduce the mammalian lipid categories FA, GL, GP, SP, and ST (Figure 1.1).

1.1.1 Fatty Acyls

Fatty acyls (FA) are carboxylic acids, which consist of a hydrocarbon chain and a terminal carboxyl group (17). They are synthesized by chain elongation of acetyl-CoA with a malonyl-CoA or methylmalonyl-CoA group (1), which imparts a hydrophobic character to the overall structure. Whereas most saturated and monounsaturated fatty acyls can be synthesized by human themselves, most polyunsaturated fatty acyls need to be obtained through diet, e.g. n-6 linoleic acid (LA) and n-3 *α*-linoleic acid (ALA) (18). Fatty acyls serve both as energy storage and building blocks for complex lipids, e.g. as components of glycerophospholipids. Their biological activities influence a range of processes and functions in living organisms, cell and tissue metabolism, and responsiveness to hormonal signals. Additionally, as components of glycerophospholipids fatty acyls regulate membrane structure and as oxidized products they control intracellular signaling pathways and gene expression (19). Through these effects, they form a key category of metabolites and influence all aspects in health and disease (19, 20). Depending on their chemical structure, fatty acyls can be divided into different subclasses, for example fatty alcohols or fatty esters. Fatty acids, which include saturated and unsaturated species, represent one of the most common subclasses of fatty acyls. Furthermore, they can be classified according to their number of carbon atoms and number, position, and stereochemistry of the double bonds. In principle, FAs are classified according to their number of carbon atoms and double bonds. Short-chain fatty acids (SCFA) contain less than 6 carbons, medium-chain fatty acids (MCFA) possess between 6 and 12 carbons, and long-chain fatty acids (LCFA) consist of more than 12 carbon atoms. The degree of unsaturation depends on the number of double bonds. FA species are described as being unsaturated when they have no double bond, whereas monounsaturated FAs contain one double bond, and polyunsaturated FAs have multiple double bonds. In mammals most common fatty acids are even numbered and saturated or monounsaturated fatty acids with chain lengths of 16, 18, and 20 carbon atoms (21). However, species with 2 to 36 carbon atoms, including odd-numbered species, can also be found in nature (22). Two examples are shown in Figure 1.2.

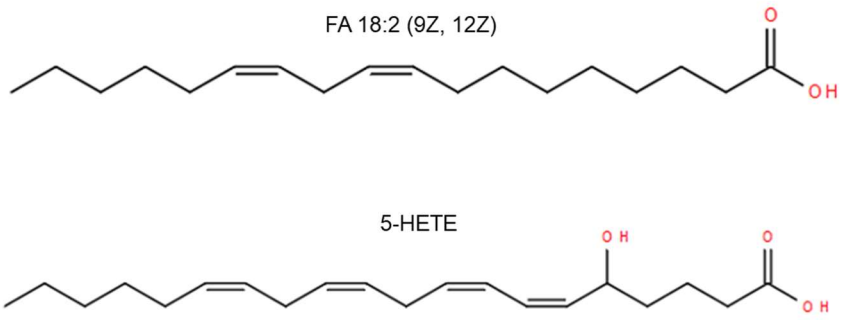


Figure 1.2: Structures of FA 18:2 and 5-HETE from LIPID MAPS®.

Catabolism of FA by *β*-oxidation converts long-chain fatty acids into two-carbon acetate which is introduced into the citric acid cycle as acetyl-coenzyme A (acetyl-CoA) for energy production (23). Fatty acid metabolism plays an important part in meeting the energy demands of the heart, for example in promoting cardiac pathologies or protecting the heart from cardiovascular disease (24). Besides that, dysregulation of the fatty acid homeostasis can lead to atherosclerosis and various types of cancer. This is often related to an uncontrolled endogenous palmitic acid biosynthesis (25).

1.1.2 Glycerolipids

Glycerolipids (GL) are a structurally heterogeneous group of lipids that can be categorized into different classes depending on the esterification of fatty acyls with glycerol. In each of these classes, several subclasses exist due to the differences in their ether or acyl linkage, thus leading to a very high number of molecules. All species have at least one hydrophobic chain which is linked to a glycerol backbone in an ether or ester linkage (26). Depending on the esterification of one, two or three fatty acyls with glycerol, the lipid classes are referred to monoacylglycerol (MG), diacylglycerol (DG), and triacylglycerol (TG), respectively (20). The structures of these lipid classes are shown in Figure 1.3.

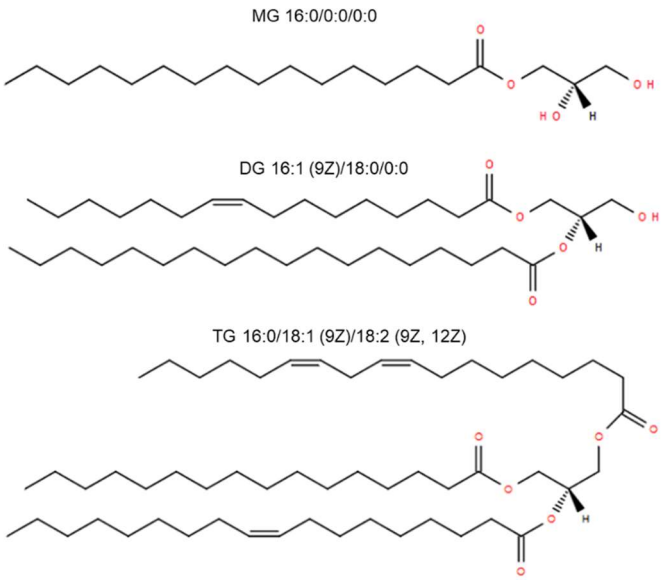


Figure 1.3: Structures of mammalian glycerolipid species MG 16:0, DG 34:1 and TG 52:3 from LIPID MAPS®.

Glycerolipids play prominent roles in physiology and human disease; they are associated with fat storage, metabolic disorders, and cancer survival (27). Whereas there is only little information available on the function of MG, DG is a neutral lipid involved in the synaptic vesicle cycle (28) and in the formation of membranes (29). Additionally, post prandial hyperlipidemia, a risk marker for cardiovascular disease, is known to be improved by DG species (30).

The chemical structure of DGs is versatile. Two (different) fatty acids are esterified to the glycerol backbone and can occur as three different stereo- or regioisomers, either at *sn-1/2*, *sn-2/3* or *rac-1/3* position, respectively (31). A schematic depiction of the different regioisomers is shown in Figure 1.4. Depending on the fatty acid species esterified to *sn-1* or *sn-3*, DGs can be achiral or chiral. They are termed as achiral when two identical fatty esters are bound to these positions. If two different fatty acids are attached to the glycerol backbone, DG is chiral (31). Due to the stereochemical nature of DG isomers, the species have different metabolic and nutritional characteristics (32). Several studies demonstrated the physiological and anti-obesity effects, in particular of 1,3 DG (33, 34). As such, DG has shown to reduce both body weight and visceral fat mass (35). Due to the ability of these lipid species to suppress both obesity and post prandial hyperlipidemia, DGs have been increasingly incorporated into food products (36, 37).

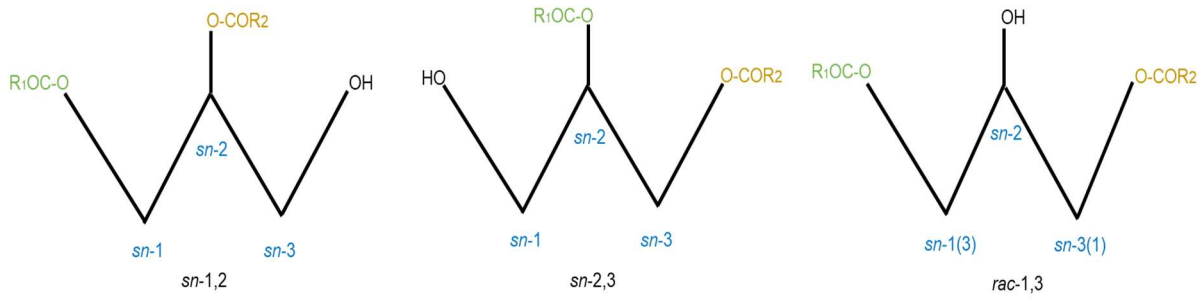


Figure 1.4: Schematic depiction of the different forms of regioisomers of diacylglycerol. Reprinted with permission from Creative Common Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Copyright Eichmann, T.; Lass, A.; 2015 (31). Illustration has been modified.

In adipose tissue of higher eukaryotes TGs serve as the major energy storage molecule (38), since they provide much more energy during oxidation than carbohydrates or proteins (39). In addition to storing energy, the synthesis of TGs protects the cell from the potentially toxic effects of excess FA. However, excessive accumulation of TGs is associated with human diseases, such as diabetes mellitus, obesity, and hepatic steatosis. Triglycerides mainly exist as triacylglycerol species in nature, however, there is also a small amount of ether-linked triglyceride species (40), which do not only differ in chain length and degree of saturation but also in chemical and physical properties (41). As already mentioned for the DG species, there are also different stereo- or regioisomers of TG. This species contain a chiral center and, therefore, optical activity, when two primary hydroxyl groups are esterified with different fatty acids (42). However, analysis of TG composition is very challenging due to the tremendous amount of individual species caused by the number of possible FA combinations on the glycerol backbone. The knowledge of the TG structure, especially the fatty acids linked with the glycerol backbone, is of great importance for understanding the lipid metabolism and e.g. the production of food products (43). Additionally, specific effects on health are obtained, which can be assessed by a deeper insight into the individual TG types. Most remarkably, there is an association between saturated TGs with short carbon chains and insulin resistance (44). Regarding TGs, many studies mainly deal with fats and oils originating from plants or animals. Composition and structure of TG species determine the functionality of these fats and oils as food ingredients and their physiological effects as part of the human diet (43). Vegetable oils provide 25% of the food calories in industrialized countries (45). Humans consume approximately 90-120 grams of fat per day. More than 95%

are absorbed (46). However, TG species cannot be absorbed directly by the gastrointestinal (GI) tract. Digestion of TG begins in the stomach. Approximately 15% of the fatty acids from TGs are released by preduodenal lipases, gastric lipase or lingual lipase (47). The absorption of dietary fats mainly takes place in the small intestine. Therefore, the fatty acids at position 1 and 3 of the glycerol backbone must be removed by pancreatic lipase. The resulting FAs and MGs can then be absorbed via active transport and/or diffusion processes by small intestinal enterocytes. After absorption FAs and MGs are then used to synthesize TGs within the enterocytes. Synthesized TGs are combined with cholesterol, cholesterol esters and apolipoprotein B48 to form chylomicrons (48, 49). Diabetes mellitus, obesity and excessive alcohol consumption can lead to an increase of TGs in the human blood, which is called hypertriglyceridemia. In rare cases hypertriglyceridemia can cause life-threating acute pancreatitis (50).

1.1.3 Glycerophospholipids

Glycerophospholipids (GPL) are amphiphilic molecules and consist of a hydrophilic head group. This head group is connected by a phosphate ester at one of the terminal positions of the glycerol backbone and one or two hydrophobic fatty acids are ether- or esterified at the remaining hydroxyl positions (51). In all living organisms, GPLs play a key role in cellular membrane and have important functional and structural properties, e.g. as cellular messengers or enzyme activators (42). Due to their different polar head groups, GPLs can be divided into different classes. The main subclasses are phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS), which are shown in Figure 1.5.

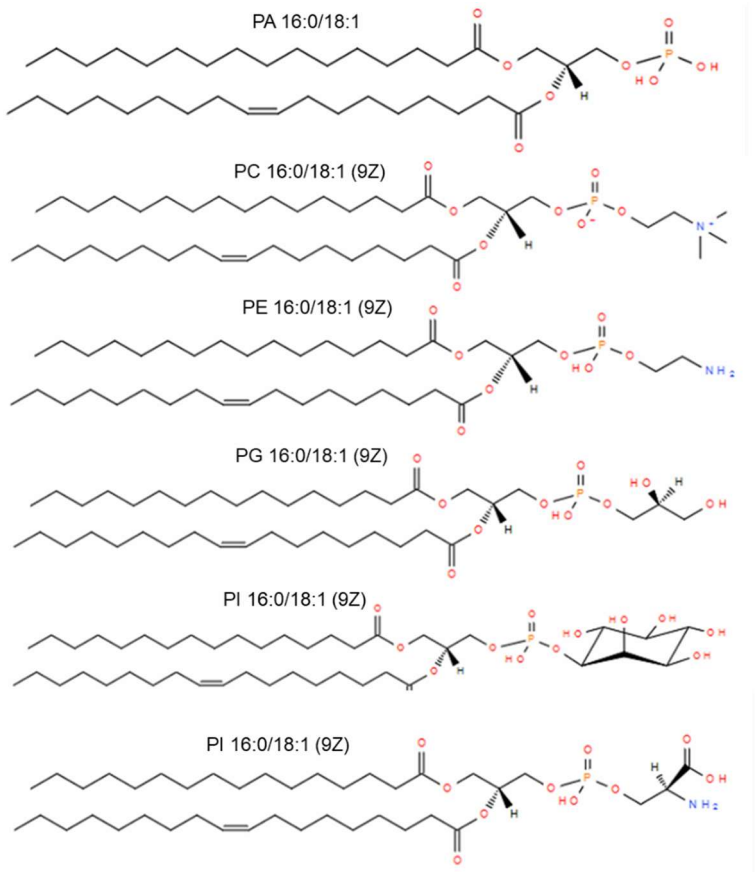


Figure 1.5: Structures of different glycerophospholipid species from LIPID MAPS®.

Glycerophospholipids are also defined by a *stereospecific numbering* (*sn*) convention, which indicates the relative positions of head group and acyl chain attachment. The modification of glycerol leads to chirality around the central carbon (52) as it is described for glycerolipids. In eukaryotic cells, the fatty acyl chains are typically attached at the *sn*-1 and *sn*-2 positions, whereas the head group is esterified at the *sn*-3 position of the glycerol backbone. In GPLs, two different fatty acyl chains allow two regioisomers with alternating substitutions at the *sn*-1 and *sn*-2 positions. Various studies showed that unsaturated fatty acyl chains were preferably esterified at the *sn*-2 position (53). However, there is increasing evidence that both *sn*-positional isomers are frequently present. Different biophysical and chemical properties of regioisomers were observed. Therefore, the research suggests that these isomers have different functions in nature (54). GPLs make up most of the lipids of cell membranes, consisting of a multiplicity of individual protein and lipid species. Usually, these membranes are composed of two layers of lipid molecules and therefore play an important role in determining the physicochemical properties. The GPL composition can be very different for diverse cell types, organelles, and inner or outer membrane leaflets,

respectively (55). The inner side of the outer membrane mainly contains PE, PG, and the respective monoacyl-glycerophospholipids *lyso*-PE and *lyso*-PG (56). The introduction of polyunsaturated fatty acids into the glycerophospholipids of the membrane is promoted by the deacylation-reacylation cycle. This process is also known as Lands-Cycle and includes the following enzymes: phospholipase A_2, acyl-CoA lysophospholipid acyltransferase, acyl-CoA synthetase, and acyl-CoA hydrolase (57).

1.1.4 Sphingolipids

The lipid class of sphingolipids (SP) is defined by a long chain sphingoid base which may be linked to a fatty acid at C2 position via an amide bond. Sphingoid base d18:1 is the most common of these backbones in mammalian cells. It varies in chain length, degree of saturation (58, 59) and number of hydroxyl groups (60), e.g. sphinganine ((2S,3R)-2-aminooctadecane-1,3-diol, which is fully saturated, and phytosphingosine ((2S,3S,4R)-2-aminooctadecane-1,3,4-triol), which is also fully saturated and has a third hydroxy group (58, 59).

The simplest class of SP are ceramides (Cer) which are formed by adding an acyl chain to the amino group of the sphingoid base. Sphingolipids can also be distinguished by the type of head group that replaces the hydroxyl group of the carbon in the 1-position (61). Phosphosphingolipids, for example, contain a phosphodiester bond at the head group and glycosphingolipids a β-glycosidically bonded sugar, whereas sphingomyelin (SM) is generated by the addition of a phosphocholine head group (62). Thus, sphingomyelins (SM), ceramides (Cer), and hexosylceramides (HexCer) differ by their head groups but frequently contain a dihydroxy C_{18} sphingosine base (62) (Figure 1.6).

C16-, C18-, and C24-ceramides are most commonly found in mammals. They provide a variety of unique properties (61, 63, 64), e.g. they serve as precursors to form either phosphor- or glycosphingolipids by adding diverse head groups. Ceramides are hydrophobic lipids which increase the molecular order of phospholipid-containing membranes (65) although they are only minor components within these membranes (66). In the Golgi apparatus ceramides can be converted into SM or glucosylceramide (GlcCer) (61). Sphingolipids play an important role in cell signaling and plasma membrane structure. They comprise a broad spectrum of complex lipids and represent one of the most important lipid classes in eukaryotic cells (67).

Whereas sphingosine-1-phosphate (S1P), for example, plays a crucial role in cell survival, cell migration or inflammation (68), Cers mediate many cellular stress response including the regulation of apoptosis (69) and cell senescence (70). Various biological processes are regulated by sphingolipids, including growth, invasion, migration, proliferation and/or metastasis. In the latter case, signaling function can be controlled within the cancer cell signal transduction network (71, 72). Ceramide and sphingosine formation can be induced by chemotherapy, radiation and/or oxidative stress (73). Irregular intracellular apoptotic signal transduction can cause changes in the levels of individual sphingolipids and thus it can trigger such disease states (74).

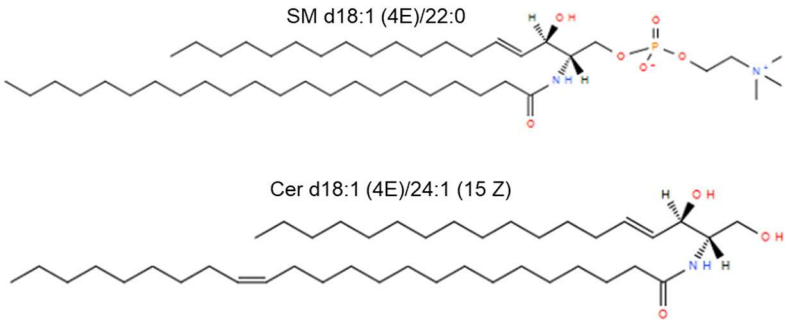


Figure 1.6: Examples of mammalian sphingolipids from LIPID MAPS®.

1.1.5 Sterol Lipids

Sterols (ST) are isoprenoid-derived amphipathic biomolecules which play important physical and structural roles in eukaryotic cells (75) and thus provide valuable insights into the evolution of life (76). All sterols have a common structure which consists of a tetracyclic cyclopenta(a)phenanthrene nucleus. Furthermore, some sterols are connected to carbon 17 via a side chain (76). Sterol synthesis involves the polymerization of the precursors isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (75).These lipids can be categorized in sterols, steroids, steroid conjugates, secosteroids and bile acids. The most common sterols in plants are phytosterols, e.g. campesterol, stigmasterol or sitosterol. In animals and yeast cholesterol and ergosterol are predominant (77) (Figure 1.7), respectively. With few exceptions (78, 79), sterols are found exclusively in eukaryotes. They are involved in various cell functions, e.g. in membrane fluidity and structure, developmental regulations or as precursors of hormone and signal molecules.

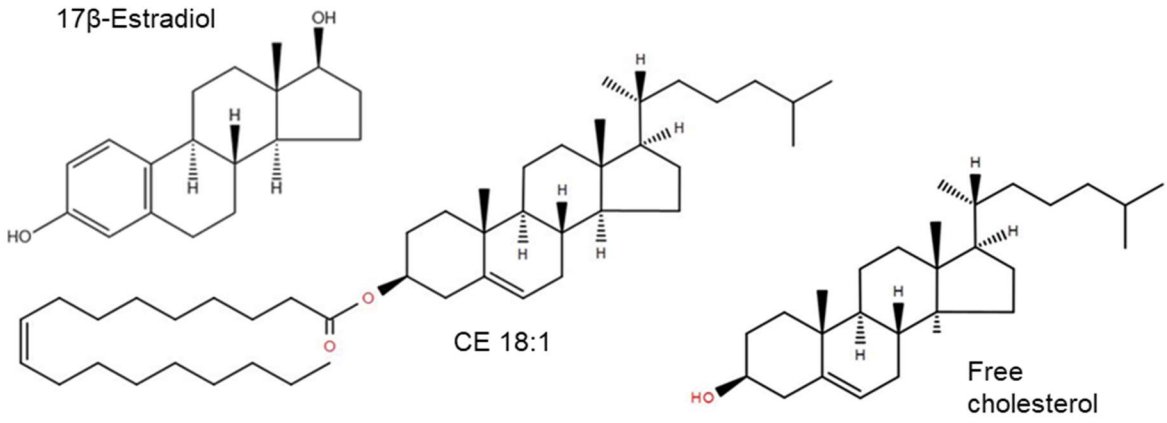


Figure 1.7: Exemplary species of sterol lipid species from LIPID MAPS®.

Cholesterol, which is especially important in mammalian cells, consists of 27 carbon atoms. Modification of the side chain or the nucleus allow a variety of different structures (76). Cholesterol occurs mainly as cholesteryl esters (CE) which are stored in lipid droplets or transported in lipoprotein particles, and as non-esterified free cholesterol (FC) in membranes (80). Cholesterol is primarily synthesized from acetyl-CoA by sequential enzymatic reactions in tissues, especially the liver, adrenal gland, brain, ovary, and testis (81). Hydrogenation of sterols leads to the formation of stanols, usually by microbes either in animal gastrointestinal tract or in the environment (82, 83). Due to their low water solubility and their ability to bind to organic material, 5*β*-stanols are particularly useful as direct biomarkers for animal feces (84). The so-called stanol fingerprint, i.e. its distribution in the fecal material, identifies a certain mammalian species on the basis of various processes, including the basis of their diet (primary sterol intake), the ability to biosynthesize endogenous sterols (secondary sterol intake) and the way how they biohydrogenate sterols and convert them into stanols with the help of bacteria of the digestive tract (85). Furthermore, a distribution of microbial cholesterol-to-coprostanol conversion in human populations is bimodal, with a minority of low metabolizers and many high metabolizers, i.e. almost complete cholesterol conversion. The efficiency of cholesterol conversion is mainly dependent on the abundance of cholesterol-lowering bacteria (86). In addition, a correlation between cholesterol-lowering activity in the human gut and the overall structure of fecal microbial community has been established (87).

1.1.6 Nomenclature

In this work, lipid nomenclature is based on a comprehensive classification system for lipids presented by the Nomenclature Committee and International Lipid Classification in 2005 (1), which was updated in 2009 (88). Practical shorthand notation of lipid structures derived from mass spectrometry (MS) approaches have been developed in 2013, which enables correct and concise reporting of data and their deposition in several databases (89). The simplest way of describing the structure is to write the total number of carbons followed by the total number of double bonds. For example, DG 36:2 describes the lipid class (DG, diacylglycerol), whereas the total number of carbon atoms is 36 and the total number of double bonds is 2 in the acyl chains. This notation is used as a sum formula and gives no information about the exact composition of the fatty acyl chains. To further specify this, various acyl combinations are possible, for example DG 18:1_18:1 and DG 18:0_18:2 without specification of *sn* positions using "_" as previously proposed (89). If the *sn* positions of the fatty acyls are known, this would be expressed with the separator "/", e.g. DG 0:0/18:0/18:2 or DG 0:0/18:2/18:0 (*sn*-1 / *sn*-2 / *sn*-3). By using high-resolution mass spectrometry, it is possible to obtain a separation between ester bonds and other bond types. An ether bond, for example, is indicated by an "O-" in front of the sum of C-atoms of fatty acyls/alkyls: DG O-36:2. The letter "P" is used for a proven O-alk-1-enyl-bond, i.e. an acid-sensitive ether bond in plasmalogens. More than one "non"-ester bond is indicated in front of the bond type as d for *di*, t for *tri*, e.g. DG dO-36:2. Positions are stated according to Δ-nomenclature in front of the functional groups. Double bond position is indicated by a number (geometry unknown) or by the number followed by the geometry (Z for *cis*, E for *trans*). There are also different abbreviations for functional groups: "Me-" for methyl branch, "O-" for keto group, and "OH-" for hydroxyl group (89).

1.2 Principles of Mass Spectrometry

Many analytical methods have been developed for accurate identification and quantification of lipid species. A common analytical technique is mass spectrometry (MS) which has been established in lipidomics in recent years.

Mass spectrometry is used to quantify known materials, to identify unknown compounds and to clarify the structure and chemical properties of various molecules. The basic principle of mass spectrometry is to separate and measure ions by their specific mass-to-charge ratio (*m/z*). These ions are detected qualitatively and quantitatively by their respective *m/z* and abundance. The instrument consists of three major components: (I) an ion source which produces gaseous ions from the substance, (II) a mass analyzer resolving the ions into their characteristic mass components according to their *m/z*, and (III) a detector system which detects the ions and records the relative abundance of each species. The most commonly applied methodologies in lipidomics involve electrospray ionization (ESI) sources and triple quadrupole analyzers (90). There are also other types of mass analyzers, for example, magnetic (B) / electric (E) sector mass analyzer, linear quadrupole ion trap (LIT), time-of-flight mass analyzer (TOF), ion cyclotron resonance mass analyzer (ICR), and orbitrap. All these mass analyzers use dynamic or static magnetic or electric fields to separate ions, and operate according to two fundamental physical laws, e.g. Lorentz force law and Newton's second law of motion (91-93). The analyzers differ by analysis speed, mass accuracy, mass range, mass resolution, and sensitivity.

Mass spectrometry allows to determine the elemental composition, molecular weight and, using MS2, position of branching and the type of substituents in the lipid structure. Due to its high accuracy, sensitivity, specificity, and throughput, MS has become a preferred method for lipid analysis.

Regarding this thesis, lipid analysis was performed by flow injection Fourier transform mass spectrometry. A hybrid quadrupole Orbitrap mass spectrometer Q Exactive is coupled to a heated electrospray ionization source and a PAL autosampler. The following chapters will describe the applied technique in more detail.

1.2.1 Electrospray Ionization

In recent years, electrospray ionization (ESI) has emerged as an important "soft" ionization technique which produces intact ions. This technique represents a sensitive, robust, and reliable method for the investigation of large, non-volatile, and thermally

unstable molecules, such as those found in biological systems, e.g. in lipids, peptides, or proteins. The samples can be measured in femto-mol quantities in microliter sample volume. High voltage is applied to spray the sample solution into the gaseous phase by the ESI needle. This process comprises three steps: (I) atomization of a fine spray of charge droplets, (II) solvent evaporation, and (III) ejection of ions from the highly charged droplets (94) (Figure 1.8). The solvent slowly evaporates, often supported by a neutral carrier gas such as nitrogen which increases the surface charge density and reduces the droplet radius. These droplets are continuously dissolved by repulsion of similar charges ("Coulomb explosion") into smaller droplets as they have reached the Rayleigh limit. Droplets are then electrically charged on the surface. Depending on charge polarity, this leads to the formation of positive or negative ions (95).

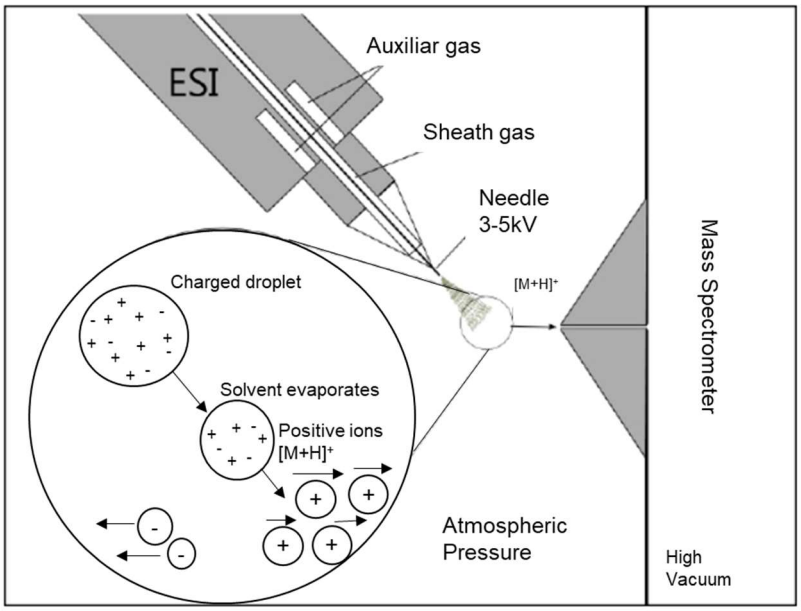


Figure 1.8: Schematic illustration of an ESI process. Reprinted with permission from Thermo Fisher Scientific. Copyright 2008 Thermo Fisher Scientific.

1.2.2 Quadrupole Mass Analyzer

One of the mass analyzers used for ESI is a quadrupole mass spectrometer which was invented by Wolfgang Paul in the early 1950s (96). The main principle of ion separation in MS is based on the movement of ions through a magnetic or electric field, whereby the movement is influenced by their *m/z* ratio. The quadrupole mass analyzer consists of four parallel hyperbolic/circular metal rods. Opposite rods are electrically connected. A direct current (DC) voltage and a radio frequency (RF) of the same amplitude and

sign are applied to the diagonally arranged rods. The rod pairs, however, differ in their polarity. By means of an electric field, the ions are moved forward in the z-direction with an oscillating movement in the x-y-plane. The applied ratio of voltages can be used to control the oscillation amplitude to allow ions of a specific *m/z* ratio to finally reach the detector. Vibration amplitudes of unwanted ions can be large and unstable. Hitting the metal rods these ions are neutralized and do not reach the detector, see Figure 1.9. Quadrupole mass analyzers are robust, economical, physically small and can be more easily connected to a variety of inlet systems such as the magnetic sector (94).

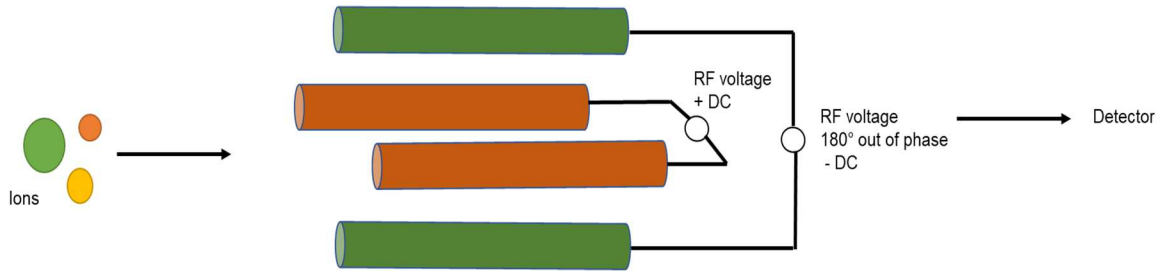


Figure 1.9: Schematic illustration of a quadrupol mass analyzer.

1.2.3 Orbitrap Mass Analyzer

The origins of the orbitrap analyzer trace back to the year 1923. Kingdon discovered the principle of orbital trapping by placing a charged wire on a closed cylindrical metal can (97). In the year 2000, Alexander Makarov published the concept of an orbital trapping device for the application in mass analysis. Unlike the previous attempts, the central electrode was not used as a thin wire but as a solid metal electrode (98). The orbitrap mass analyzer essentially consists of three electrodes: two cup-shaped outer electrodes face inwards and a spindle-shaped central electrode is aligned along the axis. The outer electrodes are electrically insulated by a hairline gap which in turn is secured by a dielectric ring. The central electrode holds the trap together and aligns it via the dielectric end-spacer. The applied electric field consists of a quadrupole field of the trap, e.g. a standard trap or a high field compact trap, and an additional logarithmic field of the cylindrical capacitor (99, 100). Applying voltage between the outer and central electrodes, stable ion trajectories induce a harmonic oscillation along the z-axis. The radial component of the field simultaneously pulls the ions towards the central

electrode. Ions with the same *m/z* ratio oscillate in-phase along the z-axis for thousands of oscillations (Figure 1.10).

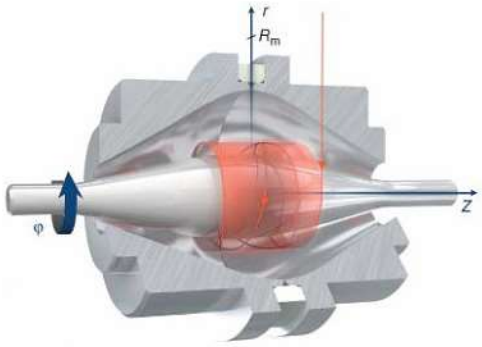


Figure 1.10: Orbitrap mass analyzer showing a stable spiral trajectory of an ion between the central electrode and the split outer electrodes. Reprinted with permission from Thermo Fisher Scientific. Copyright 2008 Thermo Fisher Scientific.

An ion trap is mounted outside the analyzer to produce stable ions. Using the principle of “electrodynamic squeezing” (101) the ions are captured and enter the trapping field by a steady increase of the electric field strength. The outer electrodes remain at a fixed potential, whereas the potential at the central electrode is lowered. Once the ions enter the field through this potential gate, they cannot escape at the point of entry, as the trapping potential forms a potential barrier until they return to the gate. To ensure the widest possible range of trapped *m/z*, a rise-time of the field, typically 30 – 50 µs, is chosen (102).

To achieve high mass resolutions orbitrap instruments apply image current detection using Fourier transform Mass Spectrometry (FTMS), using two split halves of outer electrodes for detection and a differential amplifier for amplification. The application of FT determines the frequency of the harmonic oscillations and allows the calculation of the *m/z* ratios of the subjected ions (103).

1.2.4 Tandem Mass Spectrometry

One of the most used mass analyzers in lipidomics is the triple quadrupole mass analyzer (QqQ), which is commonly used in MS/MS. It consists of three quadrupoles connected in series (Q1 – Q3). The following arrangement is most frequently used: Q1 filters for a specific *m/z* ratio (precursor ion); Q2, a collision cell, is filled with an inert gas like argon or nitrogen; and Q3 analyses the fragment ions induced by collision with

the gas molecules, shown in Figure 1.11. Both Q1 and Q3 can be used either in SIM or scan mode.

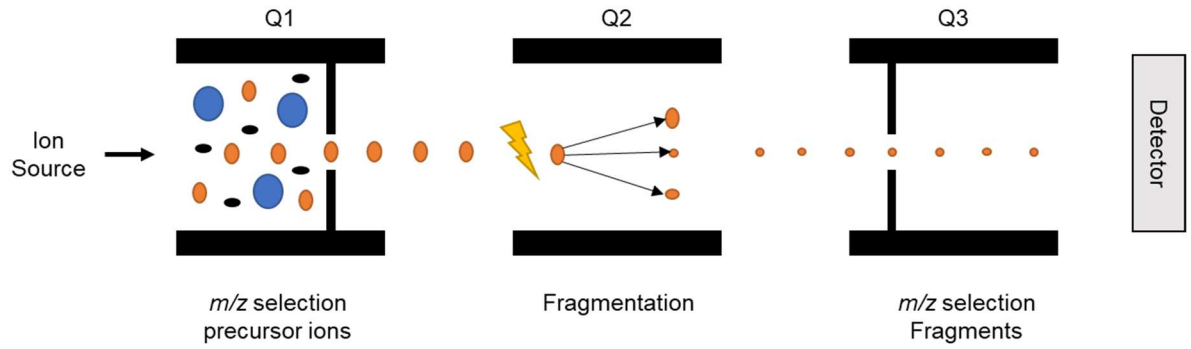


Figure 1.11: Schematic illustration of a triple quadrupole mass spectrometer (104). Modified with permission of Copyright Clearance Center's Rightslink® service. Copyright 2014 Elsevier Inc.

The following operation modes are commonly applied: Product ion scan, precursor ion scan, neutral loss scan, and selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). In a product ion scan, a precursor ion is selected in Q1, followed by collision induced fragmentation, whereas Q3 scans for resulting fragment ions. This mode provides largely substance-specific structural information. In a precursor ion scan, the precursor masses are scanned in Q1, a fragment ion is selected in Q3. Groups of substances with specific characteristic structures or fragmentation reactions can be identified. In a neutral loss mode, Q1 and Q3 are scanned with a fixed offset between both mass analyzers. It can be used to identify all precursor ions with a loss of a defined mass. In selected reaction monitoring, both mass analyzers are set to selected masses (105, 106).

1.2.5 Q Exactive Hybrid Orbitrap Mass Spectrometer

A Q Exactive Orbitrap mass spectrometer combines a high resolution Orbitrap mass analyzer and a high-performance quadrupole for precursor selection.

The Q Exactive hybrid quadrupole Orbitrap mass spectrometer mainly consists of an ion source, a stacked ring ion conductor (S-lens), a bend flatapole, a quadrupole mass filter, a curved linear trap filled with nitrogen (C-trap), a higher energy collisional dissociation (HCD) cell and an orbitrap mass analyzer (Figure 1.12).

The samples can be introduced into the ion source by various methods, while the injection flatapole transfers the ions from the source to the quadrupole. In the C-trap,

the kinetic energy of the injected ions dissipates by collision with the nitrogen molecules, bundles them on the trap axis and then injects them orthogonally to the z-axis of the orbitrap mass analyzer to obtain a mass spectrum. Furthermore, the ion bundles can be guided through the C-trap into the HCD cell and perform MS/MS experiments in combination with the quadrupole mass filter.

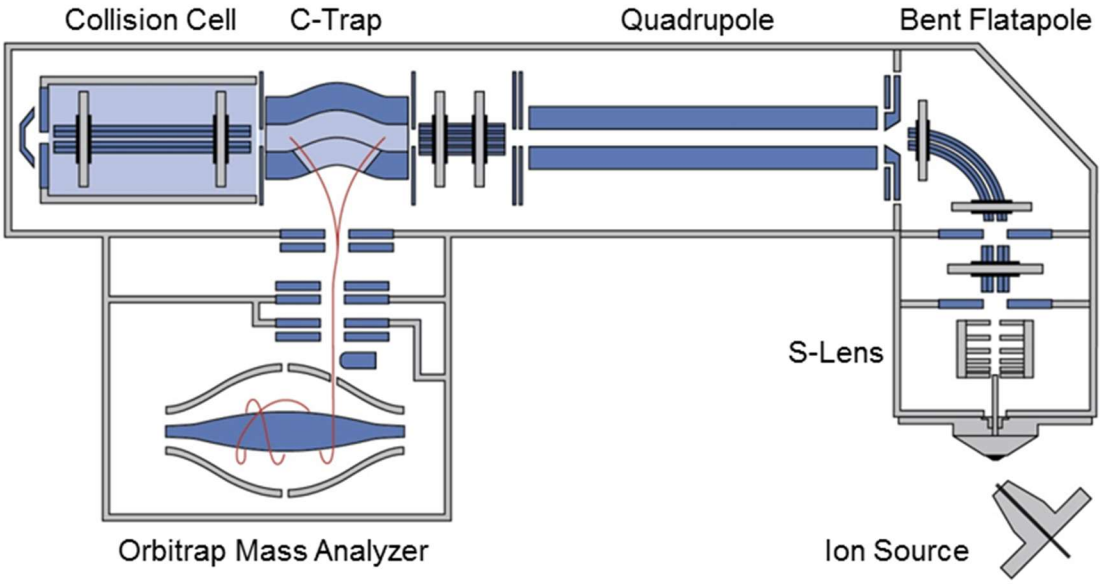


Figure 1.12: Schematic illustration of a Q Exactive Orbitrap mass spectrometer. Reprinted with permission from Thermo Fisher Scientific. Copyright 2012 Thermo Fisher Scientific.

To investigate samples in high throughput, shotgun mass spectrometry of biological samples is routinely applied (107). A commonly used technique for all lipidomic approaches is a high-resolution FTMS instrument chip-based nano-ESI (108-111). In this thesis, a Q Exactive hybrid Orbitrap mass spectrometer was used for lipid analysis, using a conventional LC pumping system to infuse raw lipid extracts of feces.

1.2.6 Direct Infusion Mass Spectrometry

In direct infusion mass spectrometry (DIMS), the sample is pumped directly into the mass spectrometer without any prior separation. Therefore, the liquid sample is placed in a syringe. A syringe pump is used to ensure a regular flow of liquid. The injection of a liquid sample into a moving, non-segmented continuous carrier stream of a suitable liquid is called flow injection analysis (FIA) (112). FIA frequently uses an HPLC system that injects the analytes directly into the ionization source of the mass spectrometer

without prior chromatographic separation (FIA-MS) (113). Flow injection is coupled to an autosampler (114).

Direct infusion is best suited for pure samples or simple mixtures and should be free of contaminating factors that could otherwise interfere with the measurement, such as high contents of non-volatile salts or detergents. Direct infusion mass spectrometers are often equipped with electrospray ionization. In this process, intact molecular masses of analytes can be detected or fragmented. This facilitates the identification of the so-called “fingerprint” of chemicals and relies on the accurately determined masses on MS level or specific fragment ions in MS/MS spectra (115, 116).

In DIMS, which is also called “shotgun”, the lipid composition and concentration of analytes does not change over time, which simplifies the quantification of the species (6). Advantages of direct infusion are its simplicity, high reproducibility, and the constant electrospray conditions, e.g. matrix, solvent composition, and sample concentration, which could influence the ionization of the analytes without significantly affecting sensitivity, precision, and accuracy (117, 118). However, disadvantages of this technique are the ion suppression effect and the inability to distinguish many isobaric or isomeric species.

1.3 Mass Spectrometric Lipid Analysis

Analysis of lipids is either based on the separation of different lipid categories by chromatographic separation or on so-called shotgun-Lipidomics which essentially analyze all lipid classes without prior chromatographic separation (119). However, a more detailed elucidation of structural properties of lipids requires the use of MS/MS analysis. This chapter describes the extraction of lipids as well as the fragmentation behavior of DG and TG with respect to the acyl chain and head group elements.

1.3.1 Extraction of Lipids

In Lipidomics it is important to perform an extraction of lipid species to obtain an accurate profile of lipidomes in a sample of interest. Each extraction method serves two main purposes. On the one hand, the complexity of the sample is reduced by eliminating unwanted non-lipid compounds such proteins. A positive side effect is the reduction of impurities, this leads to a less contaminated mass spectrometer and thus to less instrument downtime due to cleaning and maintenance. On the other hand, the lipids of interest accumulate during extraction, which in turn leads to an improved signal-to-noise ratio (16). In principle, there are two different extraction possibilities: liquid-liquid (LLE) or solid-phase (SPE) extraction. SPE is a very specific sample preparation technique and provides highly enriched samples with low contamination. However, as the number of analyzed lipid classes increases, this extraction protocol becomes very complex and challenging. If many lipid classes need to be analyzed in high throughput SPE is unsuitable. It can certainly be a useful method if only a few samples with a very high coverage of the lipid species need to be analyzed (16). In contrast, liquid-liquid extraction is the most commonly used sample preparation technique in lipidomics. Organic solvents such as chloroform/methanol ($CHCl_3$/MeOH) or methyl-tert-butyl ether (MTBE) are typically used for this purpose. Two of the most common treatments are based on the protocols by Folch (120) and Bligh and Dyer (121) and consist of a ternary mixture of chloroform, methanol, and water. Other common LLEs are MTBE extraction, single-phase extraction, butanol/methanol extraction (BUME) and 3-phase extraction. Those are listed in Table 1.1.

Table 1.1: Comparison of different LLEs regarding the extraction solvents used, the phase separation, the type of detectable lipids, and the advantages and disadvantages of the divers extraction methods (16).

	Folch	Bligh and Dyer	MTBE	Single-Phase Extraction	BUME	3-Phase Extraction
Extraction Solvents	$CHCl_3$, MeOH, H_2O	$CHCl_3$, MeOH, H_2O	Methyl-tert-butyl ether, MeOH, H_2O	MeOH; EtOH; 2-Propanol; Acetonitrile	BuOH/ MeOH, Hep/Ethyl acetate, Acetic acid	Hexane, Methyl acetate, Acetonitrile, H_2O
Bottom Phase	organic	organic	aqueous		aqueous	aqueous
Middle Phase				organic		organic (PC)
Upper Phase	aqueous	aqueous	organic		organic	organic (TG)
Detectable Lipids	saturated fatty acids	saturated fatty acids	GPL, Cer, saturated fatty acids	polar lipids	like Folch	neutral and polar lipids
Advantages	high extraction efficiency	high extraction efficiency	straightforward operation, MTBE less harmful	fast and robust	high throughput screening in 96-well plates	separate analysis runs, higher identification rate
Disadvantages	harmful due to $CHCl_3$	harmful due to $CHCl_3$		higher instrument contamination greater ion suppression effect		

1.3.2 Mass Spectrometric Analysis of Lipids

Over the last decades several mass spectrometric lipidomic methods with scientific and clinical application have been developed (12-14). A common way to reduce the complexity of lipid extracts for MS-based approaches is to use chromatographic separation, especially gas (GC) and liquid chromatography (LC) (122-124). Shotgun lipidomics, as described above, is a direct infusion-based approach without any separation prior mass spectrometric analysis. The development of high-resolution mass spectrometry led to an increasing interest in the application on MS level. The technical simplicity and rather short measurement times allow the detection of several lipid classes in a single analysis. Nowadays, shotgun MS analysis of biological samples is routinely used for high-throughput analysis of samples (107).

A detailed structural analysis of the lipid species requires MS/MS analysis using a tandem mass spectrometer. This is a very helpful and informative technique with respect to the acyl chain and head group elements for most lipid classes (125-127).

Collision Induced Dissociation (CID) of ammonium adducts $[M+NH_4]^+$ of diacylglycerol and triacylglycerol in positive ion mode results in fragment ions corresponding to the loss of 17,0266 Da (i.e. the loss of ammonia) combined with a charge-remote loss of FA moieties as fatty acids (RCOOH + NH_3), e.g. FA 16:1 has a neutral loss (NL) of *m/z* 271, FA 18:1 a neutral loss of *m/z* 299 and FA 18:0 a NL of *m/z* 301. These ions provide information about the acyl composition of both lipid classes and therefore are denoted as molecular lipid fragments (MLF) (128). For DG species a NL of water together with ammonia from an $[M+NH_4]^+$ ion is typical and denoted "-DG(35)" as lipid class selective fragment (LCF) (129) (Figure 1.13).

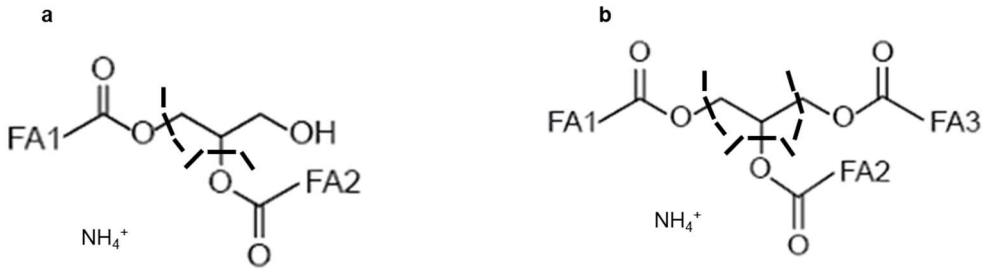


Figure 1.13: Fragmentation of diacylglycerol (a) and triacylglycerol (b) regarding their acyl chain composition. The dotted lines indicate the most labile bonds from which fragmentation most frequently occurs.
Displayed are a) DG with two acyl chains and one OH group (here 1,3 DG) and b) TG comprising three acyl (FA1 to 3) chains.

1.4 Lipid Analysis of Feces

In recent years, the interest in intestinal microbiome as well as the analysis of lipids in feces has increased significantly. The intestinal microbiome actively influences host functions and therefore plays an important role in human health and disease. Homogenization of fecal samples is of great importance for lipid analysis, as the sample material is very heterogeneous and thus more difficult to analyze. In this chapter, microbiome, feces, and homogenization are described in detail.

1.4.1 Microbiota and Feces

The human body, including the intestine, skin and other mucosal environments, is colonized by an enormous number of commensal, symbiotic and pathogenic microorganisms, collectively referred to as microbiomes, and is estimated to consist of 3×10^{13} eukaryotic cells and 3.9×10^{13} colonizing microorganisms (130). These microorganisms contain trillions of microbes with more than 700-1000 different species of bacteria in the intestine (131). It is estimated that 10 different phyla contribute to the functional role of the intestinal microbiome, with Firmicutes and Bacteroidetes being the most dominant phyla. The neutral pH and the weakly basic environment make the large intestine the most densely populated area of bacteria in the GI tract, with approximately 10^{12} bacteria/g. In the less acidic small intestine, on the other hand, only 10^{3} bacteria/g can be found (132).

It is now generally accepted that the gastrointestinal system, in particular the intestinal microbiome, actively influences host functions including nutritional responses, immunity, medication, and metabolism, and thus plays an important role in human health and disease (133). Microbial activity is reflected in fecal materials that contain unabsorbed metabolites including lipid species. Consequently, analysis of fecal metabolites provides an estimate of metabolic interaction between gut microbiota and host (134). These microbe-host interactions are impaired by the intestinal microbial ecosystem resulting from changes in lifestyle during industrialization, including important shifts in dietary habits, improved hygiene, and access to medication (e.g. antibiotics) (135). To identify subtle metabolic variations induced by dietary alterations and to characterize the metabolic impact of variations of the gut microbiota, metabolic profiling gained increasing interest over the last decade.

The intestinal microbiome can influence human behaviour through a bi-directional communication pathway between the GI tract and the central nervous system, namely

the intestinal-brain axis. This is achieved by a microbiome-mediated production of molecules, such as serotonin and *γ*-aminobutyric acid (GABA), which have neuroactive effects. Along the GI tract there are nutrient receptors and enteroendocrine cells (EECs) that interact with microbial metabolites. For mechanisms such as the regulation of appetite and insulin secretion, the interactions with EEC receptors are crucial (136). Recent studies show that bacterial proteins act locally in the intestine with a short-term effect on saturation and thus influence the appetite-regulating pathways (137).

Feces are composed of water, proteins, bacterial biomass, fat, and indigestible food components, e.g. fibres. Fat contained in feces is a heterogeneous mixture of different lipids; it constitutes 8-16% of the dry weight of the feces (138-140) and 2-8% of the wet weight (141-145). Fat found within feces originates from bacteria as well as from the undigested remains of dietary lipids (146). Non-/esterified fatty acids represent approximately 60-70% of this fat, unsaponifiable material accounts for 20-30% (147).

1.4.2 Lipid Analysis of Fecal Samples

Human feces contain, depending on diet and metabolism, different amounts of triacylglycerol (TG) and diacylglycerol (DG). This has been frequently studied in the context of steatorrhoea (148) and colon cancer (149).

Homogenization is an important step in processing tissue samples, cells or feces. Whereas homogenization is not a problem for bioliquids, it is currently still a challenge for solid sample material. Homogenization makes lipids from all parts of the sample material accessible to extraction solutions. Limited solvent accessibility of the samples can result in significantly distorted lipid profiles (16). The homogenization of feces is still difficult at this stage. An accepted protocol does not exist yet. Different working groups vortex the sample material in phosphate buffer saline (PBS) (150), whereas others homogenize for example with the help of bead beating (151). Homogenization is a prerequisite for optimal lipid extraction and is often performed manually (152). Lipidomic methods nowadays offer a wide range of possibilities to analyze lipid species profiles of biological materials (153). However, only a few methods are available to study the lipidome of fecal material (134, 154, 155). Most of the described approaches focus on the identification and quantification of selected lipid classes like fatty acids (156, 157), bile acids (158), and sterols (124). In this thesis we evaluate and validate a method for identification and quantification of DG and TG species of human fecal

material using flow injection analysis (FIA) coupled to Fourier transform mass spectrometry (FIA-FTMS).

1.5 Objective of this Work

Main goal of this PhD thesis was to develop a method for the identification and quantification of lipid species of human fecal material using flow injection analysis (FIA) coupled to Fourier transform mass spectrometry (FIA-FTMS). Method development included sample homogenization, lipid extraction, instrumental method, data validation, and data evaluation in an untargeted approach. Various homogenization methods were tested. The stability of fecal material after sample collection and the influence of material consistency were evaluated. Fecal homogenates were subjected to lipid extraction according to the protocol by Bligh and Dyer. A Q Exactive hybrid Orbitrap high resolution mass spectrometer was used for lipid analysis, equipped with a conventional LC isocratic pumping system coupled to a heated electrospray ionization (ESI) source to infuse raw lipid extracts of feces.
Method validation included limit of quantification, linearity, evaluation of matrix effects, recovery, and reproducibility. Data evaluation contained the identification of detected species using MS2 spectra.

The developed method should be accurate and reproducible as well as fast to allow high throughput of samples and to be applicable to fecal samples of clinical and scientific studies.

2. Materials and Methods

2.1 Chemicals

Methanol, ethanol absolute (EMSURE), and acetonitrile were obtained from Merck (Darmstadt, Germany), chloroform, 2-propanol, and hexane from Roth (Karlsruhe, Germany). All solvents were HPLC grade. Ammonium formate, methylamine solution 40 wt % in H_2O, methylamine solution 33 wt % in EtOH, trimethylamine solution 45 wt % in H_2O, trimethylamine solution 31-35 wt % ~ 4.2 mol/L in EtOH, sodium dodecyl sulfate (SDS), acetyl chloride of the highest analytical grade available, and a certified fatty acid methyl ester (FAME) mix (Supelco 37 component FAME mix) were ordered from Sigma-Aldrich (Taufkirchen, Germany) and isooctane (2,2,4-trimethylpentane) > 99% from Honeywell (Seelze, Germany). All chemicals and standards were of high purity grade for analysis (> 95%).

Glycerolipid standards were purchased from Larodan (Solna, Sweden): Diarachidin (DG 20:0/20:0), Dinonadecanoin (DG 19:0/19:0), Dilinolenin (DG 18:3/18:3), Dilinolein (DG 18:2/18:2), 1,2-Distearin (DG 18:0/18:0), Triarachidin (TG 20:0/20:0/20:0), Trinonadecanoin (TG 19:0/19:0/19:0), Trilinolein (TG 18:2/18:2/18:2), Triolein (TG 18:1/18:1/18:1), 1,2-Olein-3-Stearin (TG 18:1/18:1/18:0), 1,2-Stearin-3-Olein (TG 18:0/18:0/18:1), Triheptadecanoin (TG 17:0/17:0/17:0), and Tripalmitin (TG 16:0/16:0/16:0).

Furthermore, FA and MG internal standards were purchased from Larodan. All other internal standards used for quantification, LPA, PA, LPC, PC, LPE, PE, PG, PS, Cer, HexCer, and SM were ordered from Avanti (Alabaster, Alabama, USA), while PI was obtained from Christoph Thiele.

Water (aqua ad iniectabilia) for sample homogenization was purchased from B. Braun (Melsungen, Germany). Purified water was produced by Millipore Milli Q UF-Plus water purification system (Molsheim, France).

2.2 Stock Solutions

Lipid species were quantified by addition of non-endogenous internal standards. All diacylglycerol and triacylglycerol standards were dissolved in isooctane/isopropanol (3:1 v/v) at a concentration of 1.0 mg/mL. The internal standard (IS) solution contained trinonadecanoin, triheptadecanoin, and diarachidin each at a concentration of 10 µg/mL in chloroform/methanol (9:1 v/v). In previous experiments the internal

standard mixture included monoacylglycerol and fatty acyl standards. Both lipid species were adjusted to a concentration of 1.0 mg/mL. Whereas free fatty acyls were dissolved in pure methanol, monoacylglycerols were dissolved in isooctane/2-propanol (1:1 v/v). In addition to DG and TG standards mentioned above, the standard solutions for the initial tests included also monotridecanoin, mononononadecanoin, tridecanoic acid and tricosanoic acid each at a concentration of 10 µg/mL. Comparison of different solvents used in negative ion mode was performed by adding an internal cell standard, which is shown in Table 2.1. The spiked volume added to all samples during extraction was 50 µL.

Table 2.1: Internal standard used for negative ion mode experiments.

Species	**MW**	**Cell IS**	
Unit	[g/mol]	[ng/spike]	[nmol/spike]
PC 28:0	677.50	1250	1.845
PC 44:0	901.75	1250	1.386
LPC 13:0	453.29	50	0.110
LPC 19:0	537.38	50	0.093
PE 28:0	635.45	500	0.7868
PE 40:0	803.64	500	0.6222
LPE 13:0	411.24	50	0.1216
PS 28:0	679.44	750	1.1038
PS 40:0	847.63	750	0.8848
PG 28:0	666.45	125	0.1876
PG 40:0	834.63	125	0.1498
PI 34:0	838.56	250	0.2981
Cer 32:1;2	509.48	50	0.0981
Cer 35:1;2	551.53	50	0.0907
HexCer 30:1;2	643.50	60	0.0932
HexCer 35:1;2	713.58	60	0.0841
SM 30:1;2	646.50	500	0.7734
TG 51:0	848.78	900	1.0603
TG 57:0	932.88	900	0.9648
DG 28:0	512.44	250	0.4879
DG 40:0	680.63	250	0.3673
MG 13:0	288.23	250	0.8674
MG 19:0	372.32	250	0.6715
FA 13:0	214.19	250	1.1672
FA 23:0	354.35	250	0.7055

2.3 Laboratory Equipment

Table 2.2: Laboratory Equipment at the University Hospital Regensburg.

Centrifuge Megafuge 1.0R	Heraeus (Hanau, Germany)
Eppendorf Tubes	Eppendorf AG (Hamburg, Germany)
Glass Centrifuge Tubes	Hecht-Assistent (Sondheim, Germany)
Feces catcher	MED+ORG (Schwarzwald-Baar-Kreis, Germany)
Filtration Milli-Q UF Plus	Merck Millipore (Darmstadt, Germany)
GCMS-QP2010	Shimadzu (Kyoto, Japan)
GentleMACS Dissociator	Miltenyi Biotec (Bergisch Gladbach, Germany
GentleMACS 10 mL Tubes	Miltenyi Biotec (Bergisch Gladbach, Germany
PARAM Fecal analysis Tube	Sarstedt AG & Co. KG (Nümbrecht, Germany)
PAL autosampler	CTC Analytics (Zwingen, Switzerland)
Precellys homogenizer	Bertin Instruments (Montigny-le-Bretonneux, France)
Precellys lysing kit	Bertin Instruments (Montigny-le-Bretonneux, France)
Pyrex Culture Tubes	SciLabware Ltd (Riverside, UK)
Q Exactive Orbitrap	Thermo Fisher Scientific (Bremen, Germany)
Quattro Ultima MS	Micromass Communications Inc (Manchester, UK)
Sample Vials (1.5 mL volume)	VWR (Darmstadt, Germany)
Screw Caps (PTFE naturelle)	VWR (Darmstadt, Germany)
Screw Caps (PTFE, Sil, PTFE)	VWR (Darmstadt, Germany)
SpeedVac	Christ (Osterode, Germany)
Sarstedt 15 mL Tubes	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Thermomixer	Eppendorf AG (Hamburg, Germany)
Tecan Genesis RSP 150	Tecan Group Ltd (Männedorf, Switzerland)
Ultimate 3000 isocratic pump	Thermo Fisher Scientific (Waltham, MA, USA)
Ultrasonic Desintegrator	B.Braun Melsungen (Melsungen, Germany)
Ultrasonic Bath Sonorex	Bandelin (Berlin, Germany)
Vortex Genie 2	Bender & Hobein (Zurich, Switzerland)

2.4 Samples

Human fecal material was obtained from healthy volunteers for method development. Polypropylene tubes were used for sample collection. The material was gathered in the morning, immediately stored at -20°C and transported to the laboratory on ice. Samples were stored at -80°C until further processing. Samples used to investigate the influence of stool grade were collected as described by Kjølbæk et al. (159). This trial was registered under ClinicalTrials.gov Identifier no. NCT02215343.

Human plasma samples were collected from residual patient material after clinical routine diagnostics.

2.5 Sample Preparation

2.5.1 Feces Homogenization

A pea-sized, randomly selected part of the raw fecal material was homogenized in 2.5 mL isopropanol/water (70/30, v/v) using a gentleMACS™ Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described previously by Schött et al. (124). The homogenate was further diluted in 2.5 mL of 70% isopropanol and again homogenized. Homogenization was repeated twice. After vortexing briefly, 1.0 mL of this mixture was vacuum dried overnight to determine the dry weight (dw) of the raw fecal homogenate. The samples were diluted to a final concentration of 2.0 mg dry weight/mL (mg dw/mL) for further analysis. Samples were always kept on ice and stored at -80°C until further processing.

To perform the pre-analytical tests the raw feces were first homogenized in 10.0 mL water (B. Braun). The homogenization was repeated twice and the samples were kept on ice between each preparation step. After homogenization 70% of an organic solvent (e.g. methanol, ethanol or isopropanol) or water was added to 30% homogenate in a 15 mL Falcon tube. From this mixture, 1.0 mL each was divided into 1.5 mL Eppendorf tubes and stored under different conditions. The samples were kept either at room temperature (RT) or in the refrigerator at 4°C. Reference samples were immediately stored at -80°C. After defined times (1 h, 4 h, 24 h, and 4 days) the remaining samples were also stored at -80°C until further processing.

2.5.2 Lipid Extraction

An amount of 50 µL of the internal standard solution (containing 0.54 nmol TG 57:0, 0.59 nmol TG 51:0, and 0.73 nmol DG 40:0) was added to a sample volume of 100 µL (2 mg dw/mL) fecal homogenate in a glass centrifuge tube before lipid extraction. Extraction occured according to the protocol by Bligh and Dyer (121). An amount of 700 µL H_2O and 3.0 mL B&D solution ($CHCl_3$/MeOH 1:2 v/v) were added to the sample material. The mixture was vortexed for 5 sec and incubated at room temperature for at least 60 min. Subsequently, 1.0 mL $CHCl_3$ and 1.0 mL H_2O were added. The mixture was vortexed for another 5 sec and centrifuged at 4000 rpm (17,860 g) for 10 min. A volume of 1200 µL of the separated chloroform phase was transferred into a 1.5 mL glass sample vial by a pipetting robot (Tecan Genesis RSP 150) and evaporated to dryness in a vacuum concentrator. The residues were dissolved in 1.0 mL chloroform/methanol/2-propanol (1:2:4 v/v/v) containing 7.5 mM ammonium formate.
For the experiments measured in negative ion mode an internal standard solution (see Table 2.1) was added to a sample amount of 100 µL fecal homogenate or 10 µL plasma. The samples were extracted as described above. An amount of 300 µL of the separated chloroform phase was removed from all samples as mentioned earlier. The residues were dissolved in either 300 µL (feces) or 700 µL (plasma) chloroform/methanol/2-propanol (1:2:4 v/v/v) containing 7.5 mM ammonium formate and MeOH/$CHCl_3$ (5:1 v/v) containing 0.005% methylamine, respectively.
Experiments in negative ion mode involved also different solvents and methylamine concentrations, which are listed in Table 2.3.

Table 2.3: Comparison of different solvents, additives, and concentrations for measurements in negative ion mode. Solvents containing methylamine were either prepared from aqueous (a) or EtOH-containing (b) solutions. Experiments using LM2 (MeOH/$CHCl_3$ (5:1 v/v)) were performed in different concentrations of methylamine solution.

	Solvent	Additive	Concentration
LM1	2-propanol/MeOH/H_2O (8:5:1 v/v/v)	ammonium formate	7.5 mM
LM2 a/b	2-propanol/MeOH/H_2O (8:5:1 v/v/v)	methylamine solution a) H_2O b) EtOH	0.005 %
LM3 a/b	MeOH/$CHCl_3$ (5:1 v/v)	methylamine solution a) H_2O b) EtOH	0.005 % 0.01 % 0.05 % 0.1 %
LM4	EtOH/$CHCl_3$ (4:1 v/v)	trimethylamine solution EtOH	0.1 %
LM5 a/b	EtOH/$CHCl_3$ (4:1 v/v)	methylamine solution a) H_2O b) EtOH	0.005 %
LM6	2-propanol/MeOH/$CHCl_3$ (4:2:1 v/v/v)	ammonium formate	7.5 mM
LM7 a/b	2-propanol/MeOH/$CHCl_3$ (4:2:1 v/v/v)	methylamine solution a) H_2O b) EtOH	0.005 %

2.6 Flow Injection Fourier Transform Mass Spectrometry

Mass spectrometric analysis of the reconstituted lipid extracts was performed by direct flow injection analysis using Fourier transform mass spectrometry (FIA-FTMS). A hybrid quadrupole Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source was coupled to a PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an UltiMate 3000 isocratic pump (Thermo Fisher Scientific, Waltham, MA, USA). The injection volume was 50 µL and a solvent mixture of chloroform/methanol/2-propanol (1:2:4 v/v/v) was delivered at an initial flow rate of 100 µL/min until 0.25 min, followed by 10 µL/min for 2.5 min and a wash out with 300 µL/min for 0.5 min. The ion-source was operated in positive ion mode using the following parameters: spray voltage 3.5 kV, capillary

temperature of 281°C, S-lens RF level 55, aux gas heater temperature of 250°C and flow rates of 58 for sheath gas and 16 for aux gas. FTMS data were recorded in positive ion mode with a maximum injection time (IT) of 200 ms, an automated gain control (AGC) of $1 \cdot 10^6$, three microscans and a target resolution of 140,000 (at *m/z* 200). Diacylglycerol was measured in a mass rage *m/z* 450 – 800 and triacylglycerol in a range of *m/z* 750 – 1200. MS2 spectra were acquired for 3 min in mass range *m/z* 450 – 1200 with a step size of 1.0008 Da and an isolation window of 1 Da with a normalized collision energy of 20%, an IT of 64 ms, AGC of $1 \cdot 10^5$, and a target resolution of 17,500.

In negative ion mode spray voltage was set to 2.5 kV. Data were acquired in three scan events. Free fatty acyls and monoacylglycerol were measured in a mass range *m/z* 150 - 450, *lyso*-glycerophospholipids and ceramides in a range of *m/z* 400 – 650, and glycerophospholipids in a range of *m/z* 520 – 960. The lower mass range was analyzed for 0.5 min (21 averaged scans), the middle mass range was analyzed for 0.55 min (22 averaged scans), and the higher mass range for 0.6 min (25 averaged scans).

2.7 Microscopy

Fecal homogenates were documented using phase contrast microscopy with 10 x magnification (Zeiss Primovert, Jena, Germany) and the ZEN 2.6 lite imaging software.

2.8 Lipid Identification and Data Processing

ALEX software (160) was used for peak assignment and offset correction of data acquired by FTMS and MS/FTMS (MS2) using a *m/z*-tolerance of ± 0.0045 Da. Peaks with mass deviation of more than 3 ppm were not considered. ALEX software operates in several steps. In the first step, the output (.raw) files were converted into txt-files. Subsequently, the peak information in the txt-files was screened for possible lipids in a non-targeted approach. This step includes information about the isotope peaks. Emass was used for the calculation of accurate masses and probabilities of isotopic peaks as described earlier (161). Adapted to the International Union of Pure and Applied Chemistry atomic isotope probabilities were applied (162).

The offset correction of the FTMS experiments was performed by averaging the mass deviation of internal standards in positive ion mode of DG 40:0, TG 51:0, and TG 57:0 and in the negative ion mode of the lipid species listed in Table 2.4 for each mass

range, see 2.6 *Flow Injection Fourier Transform Mass Spectrometry*. A tolerance of $\Delta m/z$ 0.01 was selected. Regardless of the selected *m/z* tolerance, the area for the assigned peaks was determined from baseline to baseline.

Table 2.4: Internal standards used for offset correction in the ALEX software.

	Species	**Adduct**	***m/z***
positive ion mode	DG 40:0	$[M + NH_4]^+$	698.6657
	TG 51:0	$[M + NH_4]^+$	866.8171
	TG 57:0	$[M + NH_4]^+$	950.9110
negative ion mode	LPC 13:0	$[M + HCOO]^-$	498.2837
	PC 28:0	$[M + HCOO]^-$	722.4978
	LPE 13:0	$[M - H]^-$	410.2313
	PE 28:0	$[M - H]^-$	634.4453
	PI 34:0	$[M - H]^-$	837.5499
	PG 28:0	$[M - H]^-$	665.4399
	PS 28:0	$[M - H]^-$	678.4352
	Cer 32:1;2	$[M + HCOO]^-$	554.4790
	HexCer 30:1;2	$[M + HCOO]^-$	688.5005
	SM 30:1;2	$[M + HCOO]^-$	691.5032
	FA 13:0	$[M - H]^-$	213.1860
	MG 13:0	$[M - H]^-$	287.2228

Species assignment included evaluation of product ion spectra (see Figure 3.3). The assigned data were exported to Microsoft Excel 2010 and processed using self-programmed macros. For accurate quantification intensities were corrected for Type I isotope effects (relative isotope abundance, (163)). Type II corrections (overlap mainly resulting from ^{13}C-atoms) were not required at the selected mass resolution due to peak interference (Hoering et al. Manuscript in revision). The background correction was performed with an internal standard blank, dependent on the solvent used for feces homogenization (H_2O or 2-propanol) analyzed within the same batch. The concentration of lipid species detected in the IS blanks were subtracted from the corresponding sample. Quantification was performed by normalization of analyte to internal standard intensities multiplied with the spiked amount of the internal standard

as described recently (164). Lipids were annotated as sum composition of acyl chains or without specification of *sn*-positions using "_" as previously proposed (89).

2.9 Method Validation

Method validation of FIA-FTMS included the determination of limit of quantifiaction (LoQ), intra-day and inter-day precision, linearity of quantification, and dilution integrity.

LoQ of DG and TG species was determined from serial dilutions of different human fecal samples. Non-endogenous internal standard species DG 38:0, DG 36:6, DG 36:0, TG 54:2, TG 54:1 and TG 60:0 were analyzed in fivefold. The coefficient of variation (CV) and the absolute value of trueness – 100% were determined and plotted against the concentrations. The results were fitted by a power function. LoQ was calculated representing a CV of ≤ 20% and absolute value of trueness – 100% ≤ 20%, respectively. The higher concentration of both calculations was defined as LoQ. The concentrations of the titrated species are indicated in the corresponding figures (for details see *3.1.2 Limit of Quantification*).

Intra-day precision was assessed for five different human fecal samples which were extracted five times and quantified. For inter-day precision the experiment was repeated and the same samples were extracted and measured on five different days, within 20 days between first and last measurement.

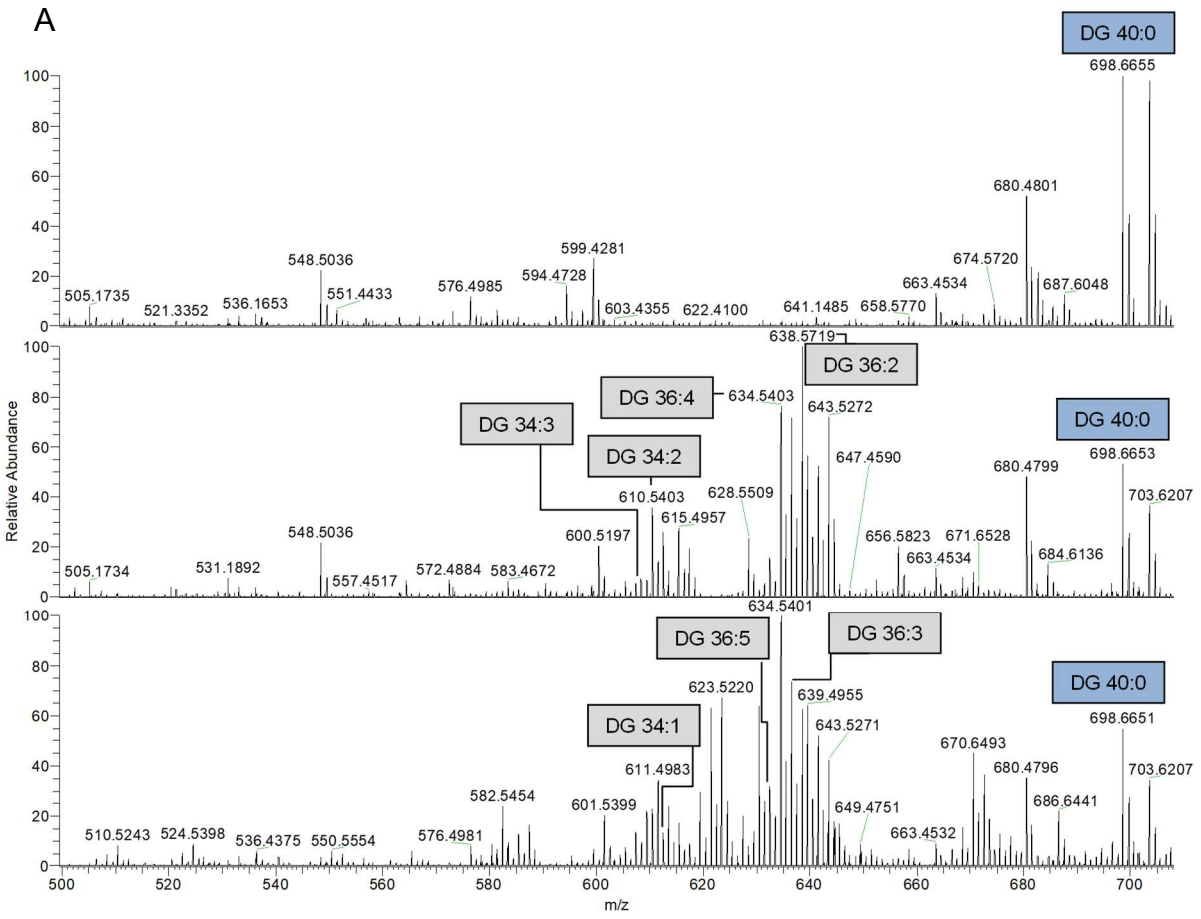
Linearity of quantification was determined using spiked samples of the synthetic standards DG 36:6, DG 38:0, TG 54:2, and TG 54:1 at six concentration levels. Each level was extracted in fivefold. The spiked concentration was plotted against the measured concentration and the results were fitted by a linear function. The experiment was performed using human fecal samples. The highest spike concentration was 8.16 pmol/mg dw for DG 36:6, 7.66 pmol/mg dw for DG 38:0, 5.64 pmol/mg dw for TG 54:2, and 5.63 pmol/mg dw for TG 54:1.

Dilution integrity of DG and TG species was determined by analysis of stool samples at different concentrations (from 1.6 mg dw/mL to 0.02 mg dw/mL). Samples were measured in triplicates. The measured quantity was compared to the target quantity determined at the highest sample concentration.

3. Results and Discussion

3.1 Lipid Species Profile of Human Fecal Samples in Positive Ion Mode

Our initial aim was to develop an accurate and fast method for the identification and quantification of lipid species in human fecal material using FIA-FTMS with a quadrupole Orbitrap hybrid mass spectrometer Q Exactive. Crude lipid extracts prepared by chloroform extraction according to the protocol by Bligh and Dyer (121) were analyzed in positive ion mode. Upon initial evaluation, spectra revealed a high heterogeneity (Figure 3.1) and numerous peaks could be assigned to $[M+NH_4]^+$ ions of DG and TG species. Other lipid classes were not detected in significant amounts. Therefore, we decided to focus on the quantification of DG and TG species.



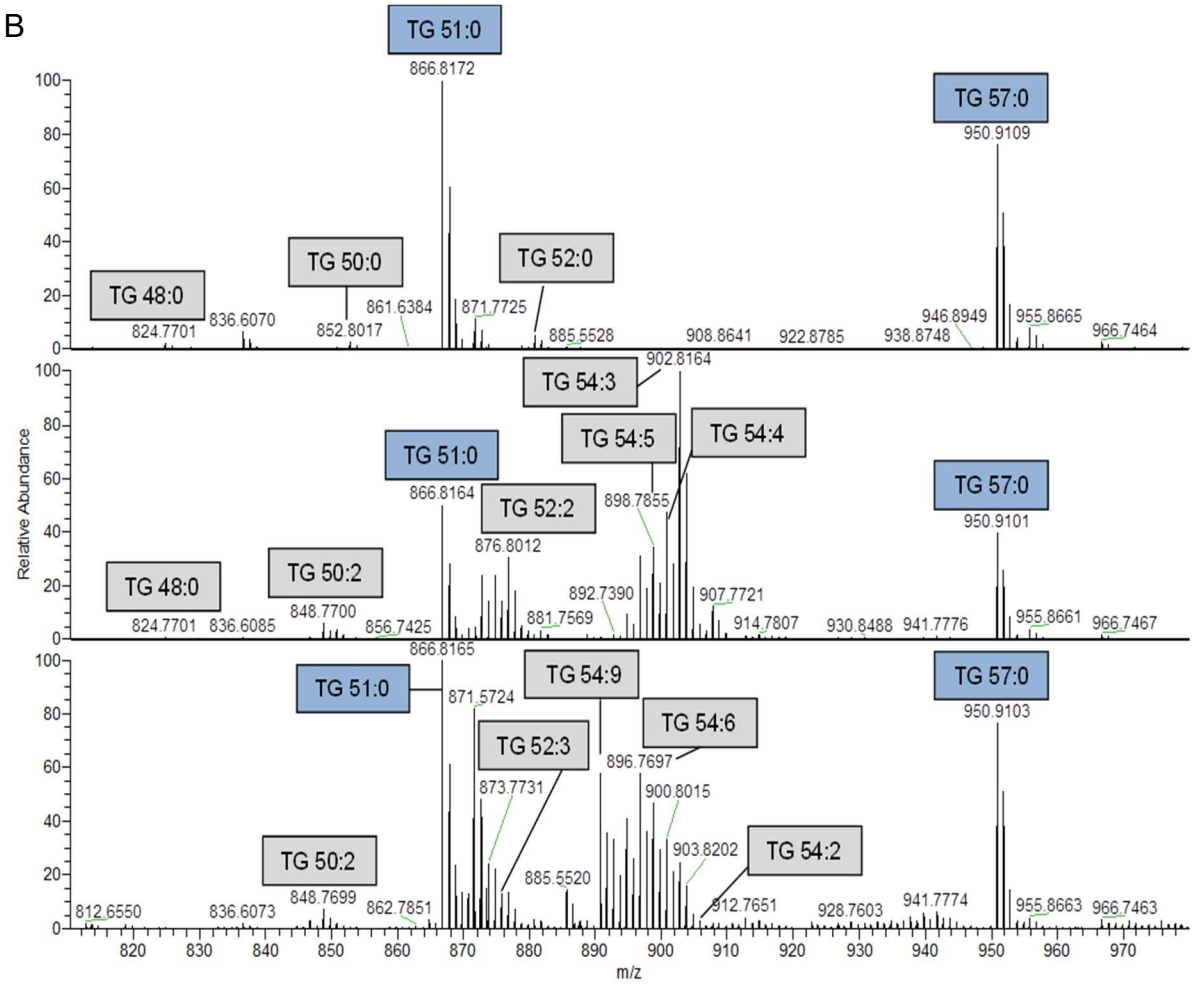
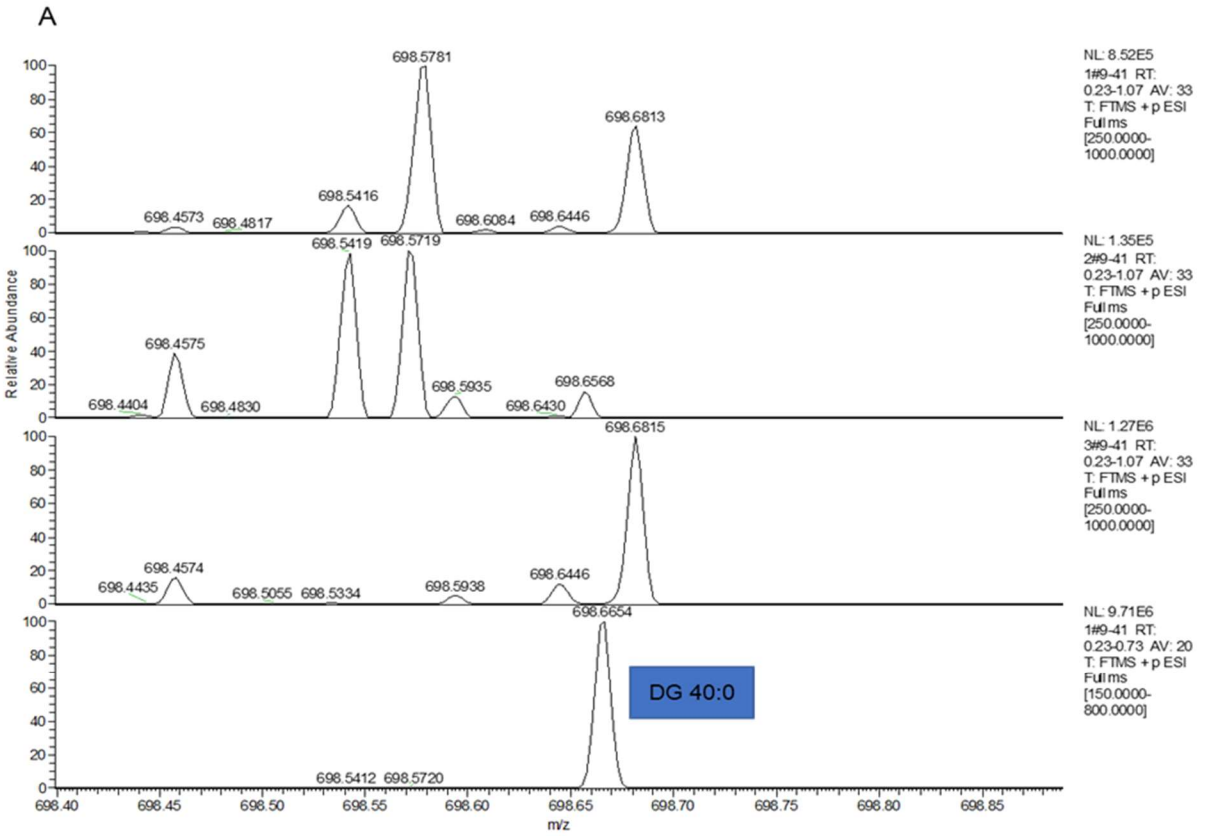
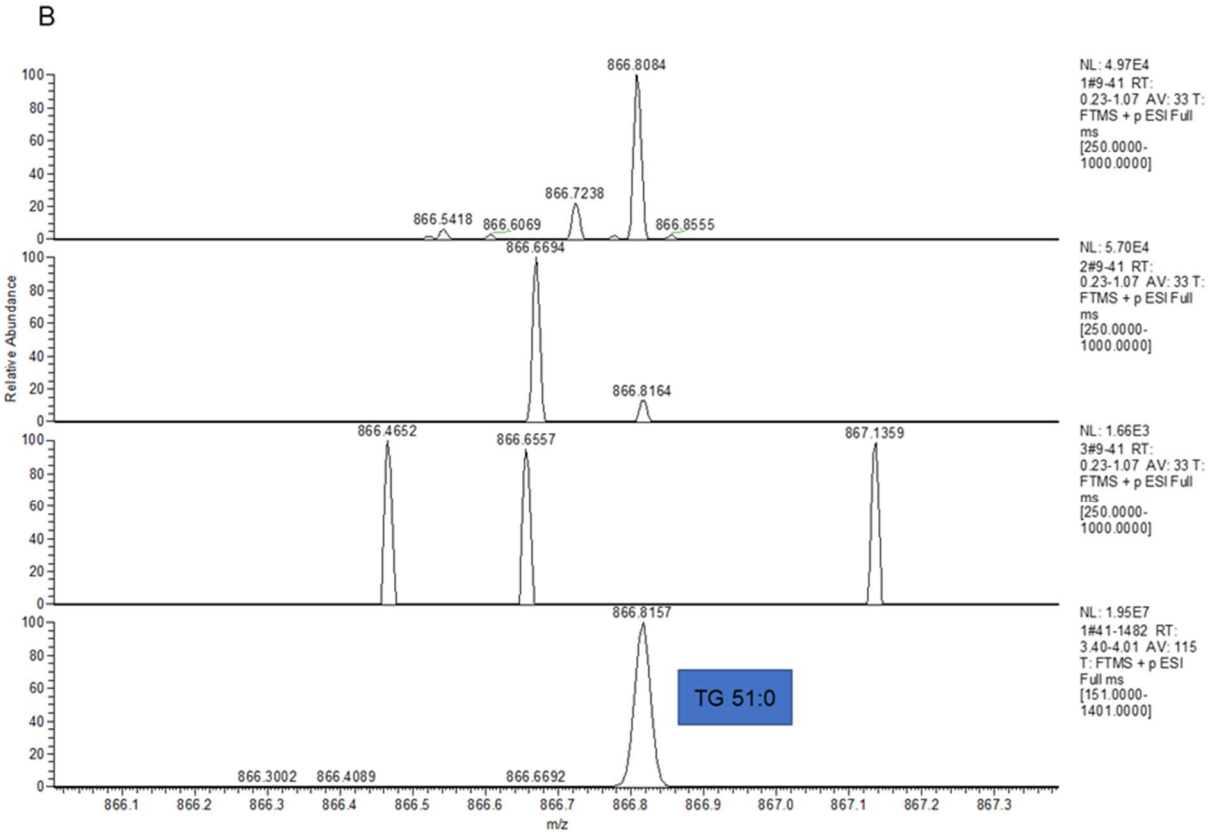


Figure 3.1: Mass spectra of three individual human fecal samples analyzed in positive ion mode are displayed. Panel A shows the mass range of DG species (*m/z* 500 – 720) and panel B of TG species (*m/z* 810 - 980). The internal standards are indicated in blue.

Quantification of lipid species was performed by adding internal standards to the sample before extraction. Ideally two lipid species which are not present in the sample material are used per lipid class.

In a first step, 20 different fecal samples were screened for their DG and TG species. None of the analyzed samples contained signals representing a relevant interference with the selected internal standards (IS) DG 40:0, TG 51:0 and TG 57:0 (Figure 3.2). DG 28:0 which is typically used for DG quantification in plasma or tissue was detectable in some fecal samples and thus could not be used as internal standard.


A
698.5781
698.6813
698.5416
698.4573
698.4817
698.6084
698.6446
NL: 8.52E5
1#9-41 RT:
0.23-1.07 AV: 33
T: FTMS + p ESI
Full ms
[250.0000-
1000.0000]
698.5419
698.5719
698.4575
698.4404
698.4830
698.5935
698.6568
698.6430
NL: 1.35E5
2#9-41 RT:
0.23-1.07 AV: 33
T: FTMS + p ESI
Full ms
[250.0000-
1000.0000]
698.6815
698.4574
698.4435
698.5055
698.5334
698.5938
698.6446
NL: 1.27E6
3#9-41 RT:
0.23-1.07 AV: 33
T: FTMS + p ESI
Full ms
[250.0000-
1000.0000]
698.6654
DG 40:0
698.5412
698.5720
NL: 9.71E6
1#9-41 RT:
0.23-0.73 AV: 20
T: FTMS + p ESI
Full ms
[150.0000-
800.0000]
Relative Abundance
698.40
698.45
698.50
698.55
698.60
698.65
698.70
698.75
698.80
698.85
m/z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866.7238
866.5418
866.6069
866.8555
NL: 4.97E4
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0.23-1.07 AV: 33 T:
FTMS + p ESI Full
ms
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1000.0000]
866.6694
866.8164
NL: 5.70E4
2#9-41 RT:
0.23-1.07 AV: 33 T:
FTMS + p ESI Full
ms
[250.0000-
1000.0000]
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866.6557
867.1359
NL: 1.66E3
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0.23-1.07 AV: 33 T:
FTMS + p ESI Full
ms
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1000.0000]
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TG 51:0
866.3002
866.4089
866.6692
NL: 1.95E7
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3.40-4.01 AV: 115
T: FTMS + p ESI
Full ms
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1401.0000]
Relative Abundance
866.1
866.2
866.3
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867.0
867.1
867.2
867.3
m/z

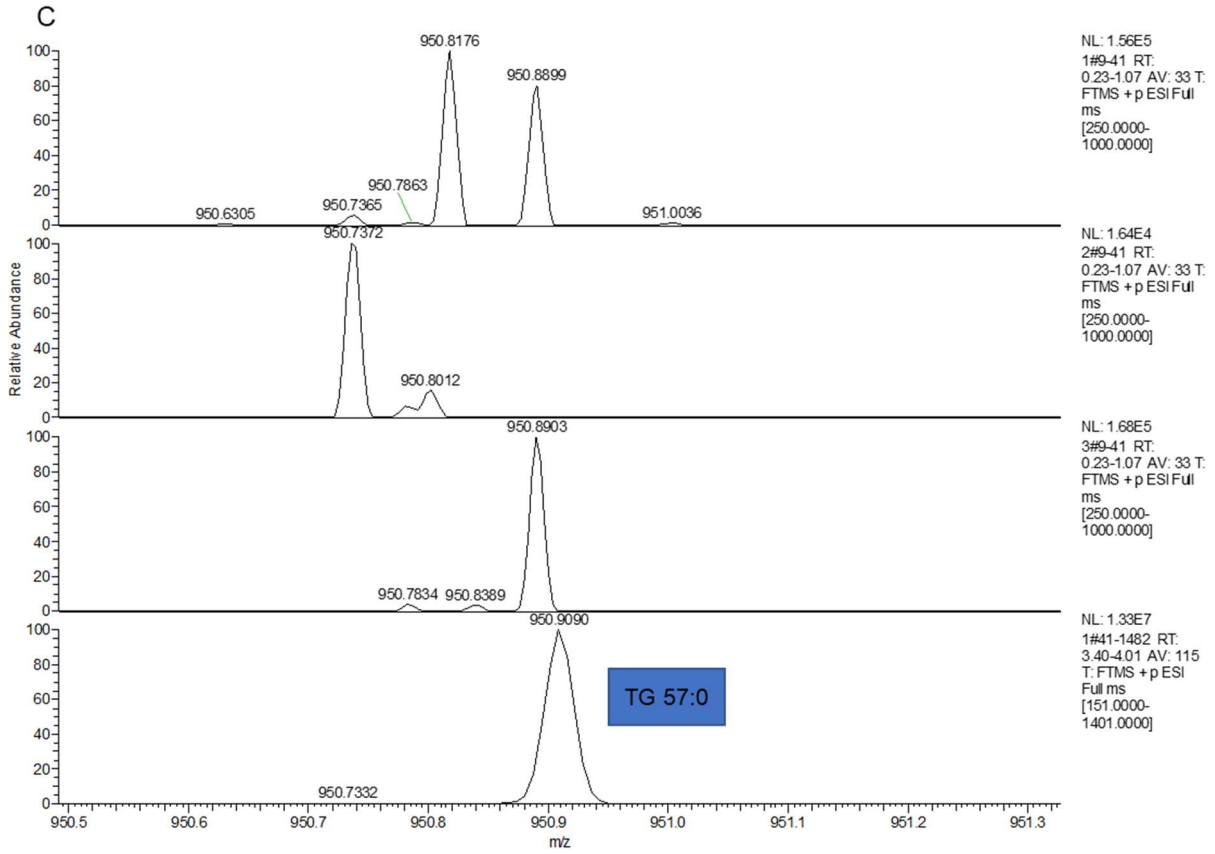


Figure 3.2: Mass spectra of four individual human fecal samples analyzed in positive ion mode are displayed. Panel A shows the mass range of DG 40:0 (*m/z* 698.6657), panel B of TG 51:0 (*m/z* 866.8171) and panel C of TG 57:0 (*m/z* 950.9110) for samples without (upper three spectra) and with internal standards (bottom spectrum).

To prove the identity of detected species, MS2 spectra were evaluated and product ions were assigned according to the annotation system proposed recently (128) (exemplified in Figure 3.3).

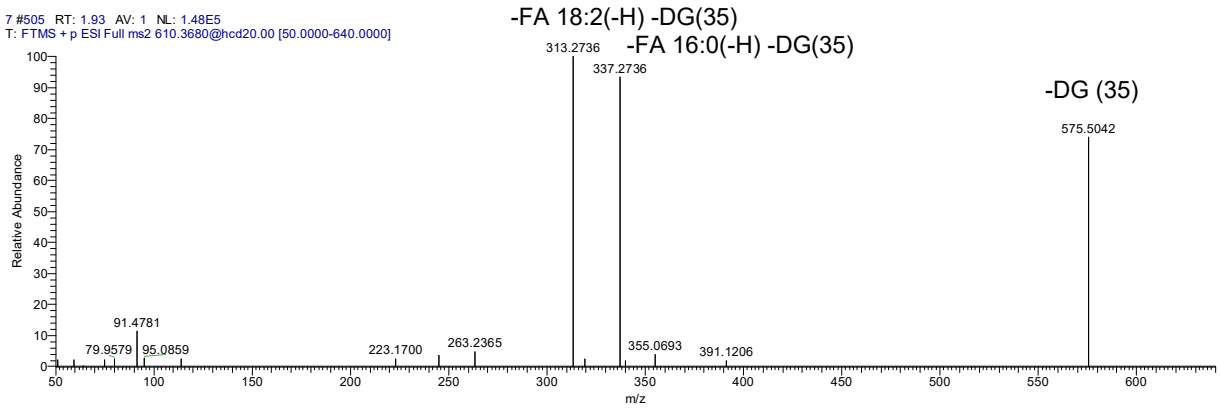
High resolution FTMS analysis detected ammoniated DG 34:2 at *m/z* 610.5405 (2.8 ppm mass accuracy). MS2 analysis of *m/z* 610.5 detected a LCF at *m/z* 575.5042, corresponding to the neutral loss of H_2O and ammonia, which is annotated as “-DG(35)”. The MLFs derived from DG 34:2 contained the fragments "-FA 16:0" at *m/z* 337.2736 and “-FA 18:2” at *m/z* 313.2736 derived from neutral losses. In addition, fragments specific to lipid species were observed for TG, for example, TG 52:4 at *m/z* 872.8, showed NL fragments: “-FA 16:0” at *m/z* 599.5028 and “-FA 18:2” at *m/z* 575.5040.

In some cases, a strong sample dependence could be observed. Whereas DG 36:2, for example, consisted of two FA 18:1 in some samples, in others following

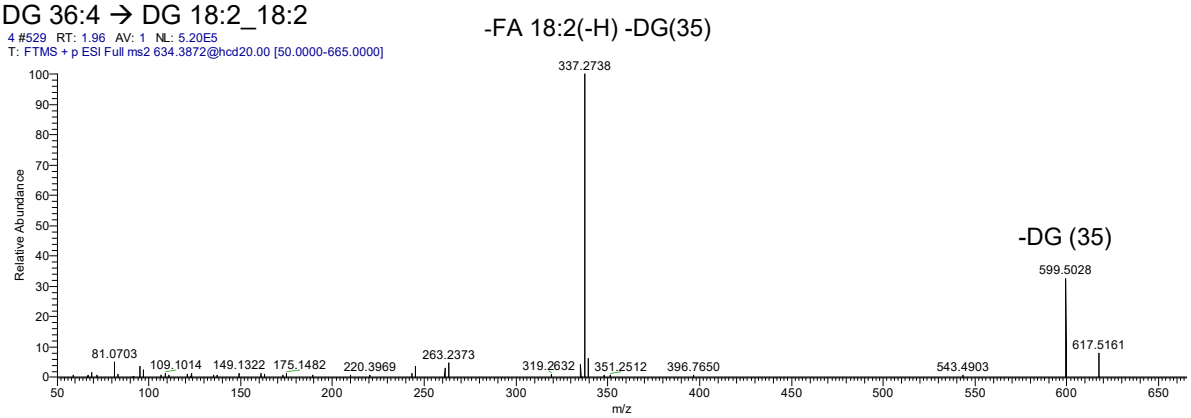
combination could be found: DG 18:0_18:2. Especially for TG it could be observed that several fatty acyl combinations occur in one sample. For example, TG 54:6 at *m/z* 896.8 revealed a NL of “-FA 18:0” at *m/z* 595.4703, “-FA 18:1” at *m/z* 597.4846, “-FA 18:2” at *m/z* 599.5009 and “-FA 18:3” at *m/z* 601.5158, with a mass accuracy better than 5.5 ppm. Thus different combinations were possible: TG 18:0_18:3_18:3, TG 18:1_18:2_18:3, and TG 18:2_18:2_18:2. Whereas “-FA 18:2” and “-FA 18:3” showed high intensities in the spectrum, these were significantly lower for “-FA 18:1” and “-FA 18:0”.

The detected NL fragments comprised mainly acyl chains with 16 and 18 carbons and up to three double bonds. For DG, also species containing FA 12:0 and 14:0 were detected precluding application of DG 28:0 as IS.

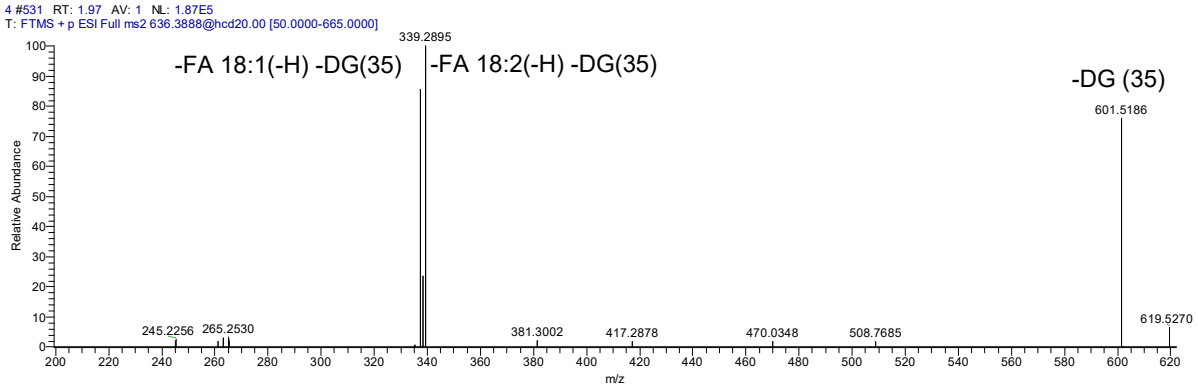
DG 34:2 → DG 16:0_18:2



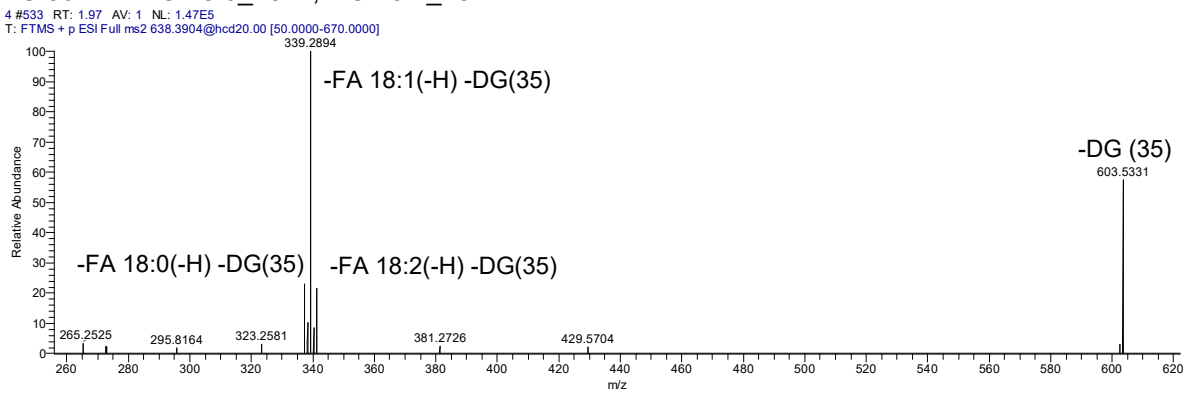
DG 36:4 → DG 18:2_18:2



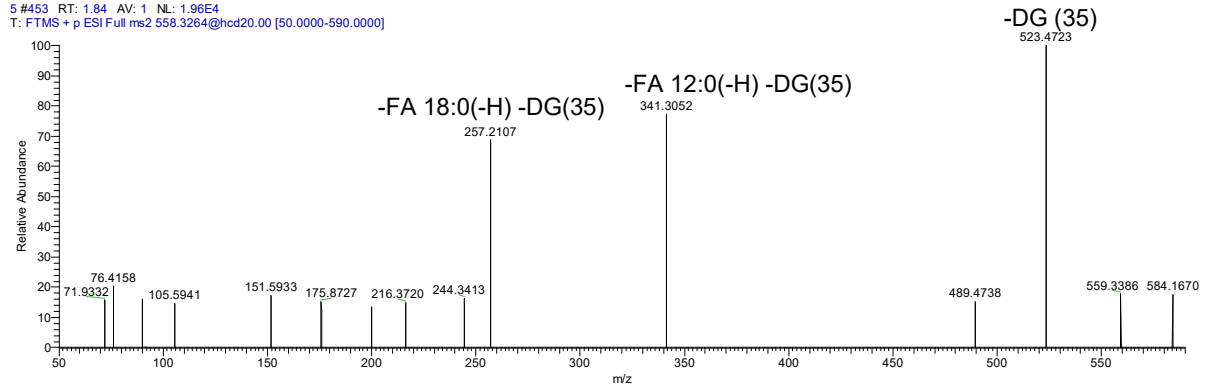
DG 36:3 → DG 18:1_18:2



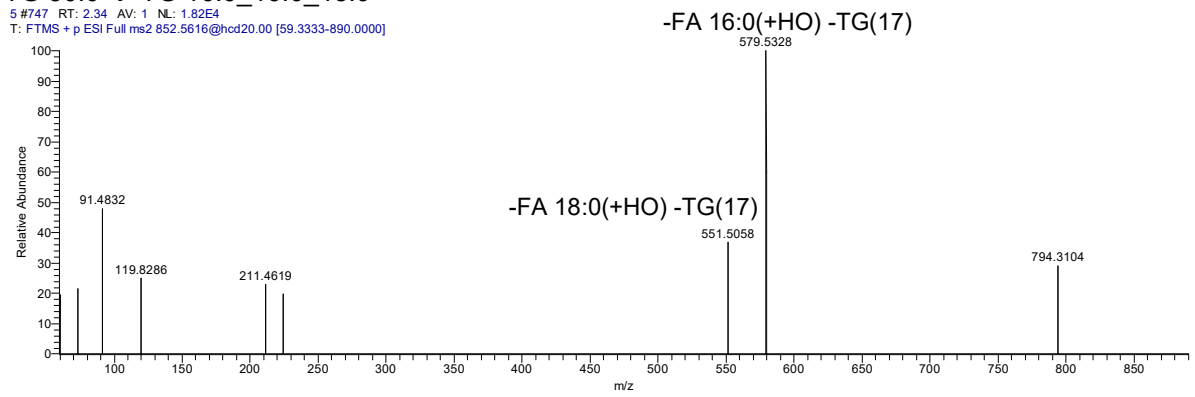
DG 36:2 → DG 18:0_18:2 ; DG 18:1_18:1



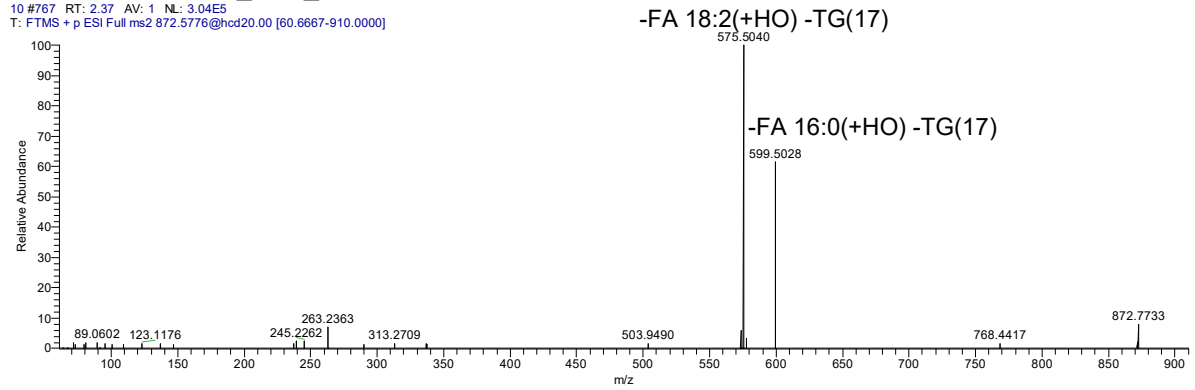
DG 30:0 → DG 12:0_18:0



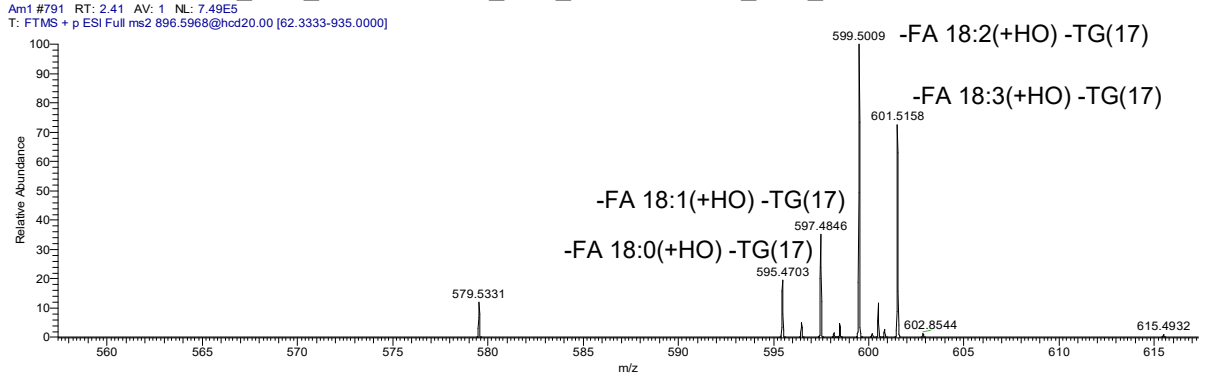
TG 50:0 → TG 16:0_16:0_18:0



TG 52:4 → TG 16:0_18:2_18:2



TG 54:6 → TG 18:0_18:3_18:3 ; TG 18:1_18:2_18:3 ; TG 18:2_18:2_18:2



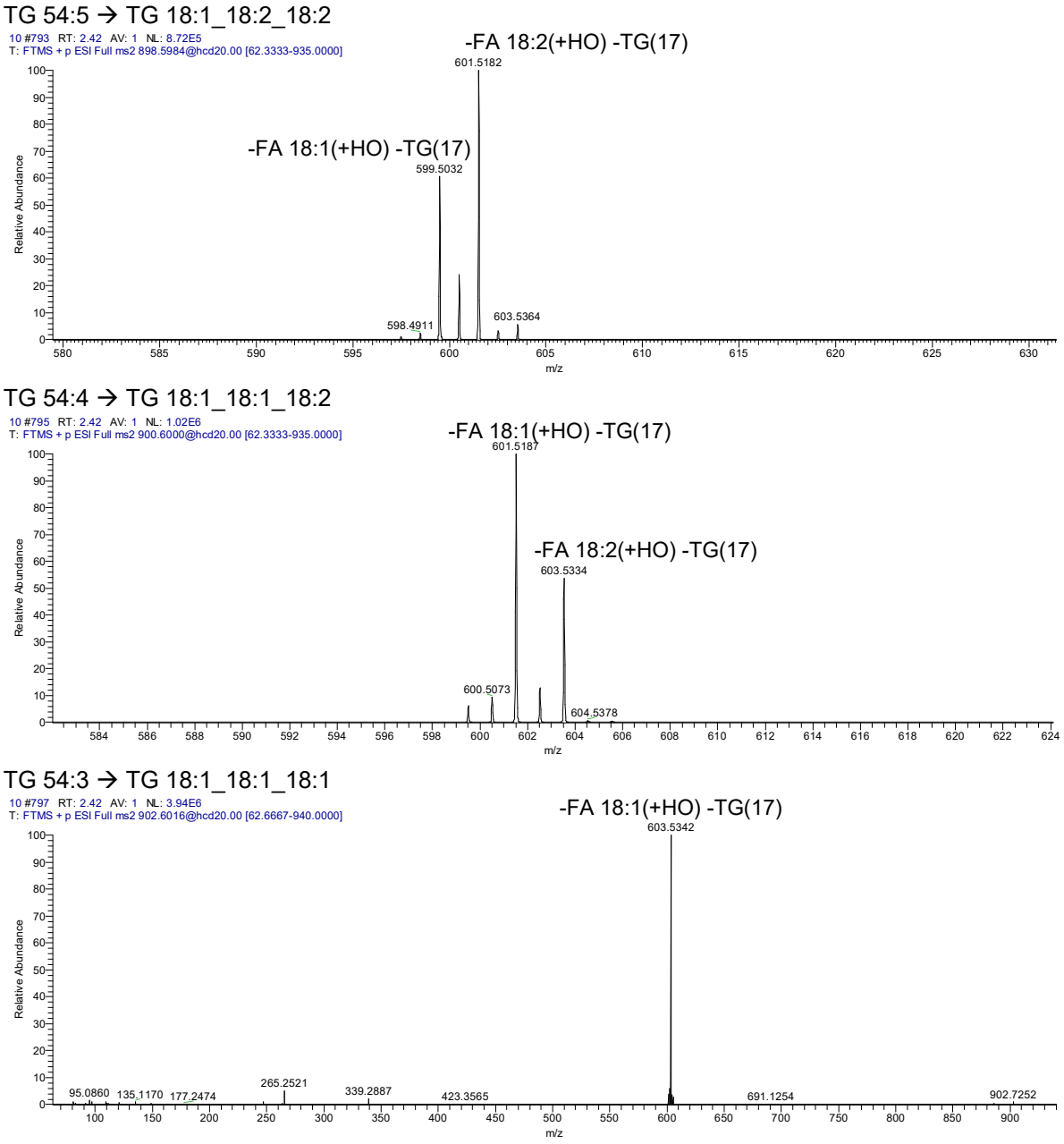


Figure 3.3: MS2 spectra of DG and TG species and acyl combinations derived from the spectra.

The concentrations of DG and TG species detected in these samples span a range up to or more than three orders of magnitude (Table 3.1). Highest mean concentrations were detected for polyunsaturated species with more than two double bonds: DG 36:3, DG 36:4, TG 54:3, TG 54:4 and TG 54:5.

Table 3.1: Concentrations and acyl combinations of DG and TG species in human feces from 20 different samples. Data based on a single measurement of the individual samples and acyl combinations were derived from MS2 spectra.

Compound	$[M+NH_4]^+$ *m/z*	mean ± standard deviation [nmol/mg dw]			median	min	max	acyl combinations
DG 26:0	502.447	0.139	±	0.348	0.004	n.d.	1.401	DG 12:0_14:0
DG 28:0	530.478	0.08	±	0.214	0.002	n.d.	0.919	DG 12:0_16:0
								DG 14:0_14:0
DG 30:0	558.509	0.106	±	0.322	0.008	n.d.	1.454	DG 12:0_18:0
								DG 14:0_16:0
DG 34:3	608.525	0.428	±	0.66	0.24	n.d.	2.808	DG 16:0_18:3
DG 34:2	610.541	1.865	±	1.856	0.994	0.045	6.198	DG 16:0_18:2
DG 34:1	612.556	0.776	±	0.711	0.503	0.068	2.417	DG 16:0_18:1
DG 36:5	632.525	1.254	±	1.922	0.726	0.002	8.481	DG 18:2_18:3
DG 36:4	634.541	7.305	±	8.349	4.302	0.053	32.355	DG 18:2_18:2
								DG 18:1_18:3
DG 36:3	636.556	4.371	±	4.513	2.452	0.042	14.508	DG 18:1_18:2
DG 36:2	638.572	3.632	±	4.283	2.564	0.074	17.947	DG 18:1_18:1
								DG 18:0_18:2
TG 48:0	824.77	0.042	±	0.104	0.01	n.d.	0.491	TG 16:0_16:0_16:0
TG 50:3	846.755	0.475	±	0.465	0.445	0.018	1.008	TG 16:0_16:1_18:2
TG 50:2	848.77	0.141	±	0.253	0.072	0.003	1.15	TG 16:0_16:0_18:2
								TG 16:0_16:1_18:1
TG 50:1	850.786	0.083	±	0.116	0.032	0.006	0.43	TG 16:0_16:0_18:1
TG 50:0	852.801	0.164	±	0.466	0.031	n.d.	2.088	TG 16:0_16:0_18:0
TG 52:5	870.755	0.105	±	0.143	0.045	n.d.	0.566	TG 16:1_18:2_18:2
								TG 16:0_18:2_18:3
TG 52:4	872.77	0.841	±	1.284	0.494	n.d.	5.869	TG 16:0_18:2_18:2
								TG 16:1_18:1_18:1
TG 52:3	874.786	0.487	±	0.901	0.174	n.d.	3.666	TG 16:0_18:1_18:2
TG 52:2	876.801	0.459	±	0.892	0.127	0.009	3.465	TG 16:0_18:1_18:1
								TG 16:0_18:0_18:2
TG 53:4	886.786	0.269	±	0.27	0.234	0.005	0.624	TG 17:1_18:1_18:2
TG 54:9	890.723	0.078	±	0.16	0.003	n.d.	0.641	TG 18:3_18:3_18:3
TG 54:7	894.755	0.409	±	0.528	0.08	n.d.	1.895	TG 18:2_18:2_18:3
TG 54:6	896.77	1.238	±	1.721	0.684	n.d.	7.388	TG 18:0_18:3_18:3
								TG 18:1_18:2_18:3
								TG 18:2_18:2_18:2
TG 54:5	898.786	1.174	±	1.886	0.599	0.003	7.875	TG 18:1_18:2_18:2
TG 54:4	900.801	1.175	±	2.25	0.422	0.003	8.988	TG 18:1_18:1_18:2
								TG 18:0_18:1_18:3
TG 54:3	902.817	1.523	±	3.239	0.294	0.008	11.56	TG 18:1_18:1_18:1
								TG 18:0_18:1_18:2

3.1.1 Reproducibility

In an important next step within method development (165, 166) we evaluated the performance of the FIA-FTMS method. Due to sample heterogeneity intra- and inter-day precisions were evaluated in five different samples (Table 3.2 and Table 3.3). The coefficients of variation (CV) were below 15% or even below 10% for most DG species. For sample 5 significantly higher variations were observed especially for TG species concentrations (see also *3.1.4 Evaluation of Reproducibility Issues*). Moreover, we observed a decrease in the concentrations of most of the TG species from day to day. Despite storage of the samples in 70% isopropanol at -80°C, this decline may be related to lipase activity since enzymatic activity has also been reported in organic solvents (167, 168).

Table 3.2: Coefficient of variation (CV) of intra- and interday precision of DG species determined in five different human fecal samples by FIA-FTMS/MS analyzed in fivefold.

Diacylglycerol	sample	Intraday Mean (n=5) [nmol/ mg dw]	CV [%]	Interday Mean (n=5) [nmol/ mg dw]	CV [%]
DG 34:3	sample 1	**0.13**	7.2	**0.13**	11.6
	sample 2	**0.36**	16	**0.38**	13.7
	sample 3	**0.61**	6.4	**0.63**	8.1
	sample 4	**0.59**	3.7	**0.53**	4.3
	sample 5	**0.12**	24.1	**0.12**	15.3
DG 34:2	sample 1	**1.45**	2.7	**1.48**	6.4
	sample 2	**3.37**	16.2	**3.53**	13.4
	sample 3	**4.32**	6.3	**4.43**	7
	sample 4	**7.55**	1.6	**4.79**	4.5
	sample 5	**1.81**	2.6	**1.75**	6.4
DG 34:1	sample 1	**1.87**	3.6	**1.92**	7.2
	sample 2	**0.67**	15.1	**0.69**	14.8
	sample 3	**1.85**	7.2	**1.89**	7.7
	sample 4	**5.66**	1.9	**5.16**	2.1
	sample 5	**0.36**	30.5	**0.33**	13.9
DG 36:5	sample 1	**0.29**	6.6	**0.29**	10.3
	sample 2	**1.09**	16.4	**1.14**	13.2
	sample 3	**1.69**	5.7	**1.74**	8.2
	sample 4	**n.d.**		**n.d.**	
	sample 5	**0.4**	4.5	**0.38**	7.2
DG 36:4	sample 1	**4.32**	3.2	**4.38**	7.5
	sample 2	**10.99**	16.2	**11.5**	13.2
	sample 3	**14.31**	6.3	**14.68**	7.3
	sample 4	**13.2**	2.3	**11.92**	4
	sample 5	**5.95**	9.6	**5.9**	9.3
DG 36:3	sample 1	**5.89**	2.3	**5.98**	6.1
	sample 2	**5.82**	14	**6.04**	11.6
	sample 3	**11.29**	6.9	**11.61**	8.1
	sample 4	**43.5**	2	**39.27**	3.9
	sample 5	**2.89**	4.6	**2.75**	5.6
DG 36:2	sample 1	**10.27**	2.2	**10.43**	6.2
	sample 2	**2.38**	14.4	**2.48**	11.7
	sample 3	**6.5**	7.7	**6.65**	7.9
	sample 4	**53.49**	2.2	**48.53**	3.2
	sample 5	**1.9**	3.2	**1.83**	5.5

Table 3.3: Coefficient of variation (CV) of intra- and interday precision of TG species determined in five different human fecal samples by FIA-FTMS/MS analyzed in fivefold.

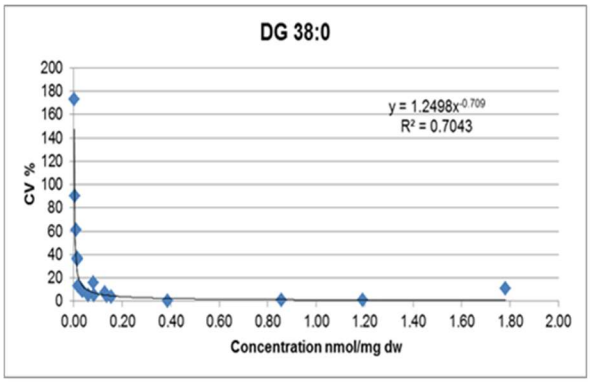
Triacylglycerol	sample	Intraday Mean (n=5) [nmol/ mg dw]	CV [%]	Interday Mean (n=5) [nmol/ mg dw]	CV [%]
TG 50:2	sample 1	**n.d.**		**n.d.**	
	sample 2	**0.13**	10.5	**0.14**	15.7
	sample 3	**4.15**	7.1	**4.15**	7.4
	sample 4	**3.5**	10.4	**3.17**	9.9
	sample 5	**0.19**	97.3	**0.11**	118.3
TG 50:1	sample 1	**0.17**	1.9	**0.18**	12.1
	sample 2	**n.d.**		**n.d.**	
	sample 3	**1.26**	5.8	**1.26**	5.9
	sample 4	**1.01**	10.5	**0.92**	9.9
	sample 5	**n.d.**		**n.d.**	
TG 50:0	sample 1	**0.22**	10.5	**0.24**	20.1
	sample 2	**n.d.**		**n.d.**	
	sample 3	**n.d.**		**n.d.**	
	sample 4	**n.d.**		**n.d.**	
	sample 5	**0.1**	7.4	**0.1**	9.9
TG 52:5	sample 1	**n.d.**		**n.d.**	
	sample 2	**0.11**	7.3	**0.11**	10.7
	sample 3	**3.41**	7.8	**3.49**	11.9
	sample 4	**2.1**	8.9	**1.91**	8.5
	sample 5	**n.d.**		**n.d.**	
TG 52:4	sample 1	**0.32**	5.9	**0.32**	14.2
	sample 2	**0.82**	7.6	**0.84**	11.6
	sample 3	**27.9**	6.9	**27.76**	7.8
	sample 4	**20.26**	9.4	**18.36**	9
	sample 5	**1.25**	87.4	**0.66**	116.8
TG 52:3	sample 1	**0.39**	9	**0.41**	16.2
	sample 2	**0.44**	13.5	**0.46**	13.4
	sample 3	**18.36**	5.1	**18.3**	5.9
	sample 4	**65.61**	10.4	**59.35**	10.1
	sample 5	**0.35**	23.6	**0.37**	140.5
TG 52:2	sample 1	**1.04**	11.1	**1.09**	14.8
	sample 2	**0.21**	10.5	**0.22**	13.5
	sample 3	**9.74**	4.9	**9.77**	6
	sample 4	**45.75**	10.8	**41.29**	10.5
	sample 5	**0.35**	92.8	**0.18**	129.3
TG 54:7	sample 1	**n.d.**		**n.d.**	
	sample 2	**0.24**	7.1	**0.24**	9.8
	sample 3	**14.67**	8.6	**15.1**	14.7
	sample 4	**0.21**	13.9	**0.19**	13.4
	sample 5	**n.d.**		**n.d.**	

Triacylglycerol	sample	Intraday Mean (n=5) [nmol/ mg dw]	CV [%]	Interday Mean (n=5) [nmol/ mg dw]	CV [%]
TG 54:6	sample 1	**0.35**	6	**0.38**	28.8
	sample 2	**1.24**	9.7	**1.27**	11
	sample 3	**51.78**	5.6	**51.45**	6.7
	sample 4	**20.86**	9.2	**18.89**	8.9
	sample 5	**1.4**	74.4	**0.77**	104.8
TG 54:5	sample 1	**0.82**	9.5	**0.85**	18.9
	sample 2	**1.41**	10.9	**1.45**	10.9
	sample 3	**52.48**	4.1	**52.47**	5.6
	sample 4	**106.95**	10.4	**96.83**	10
	sample 5	**1.85**	102.5	**0.92**	146
TG 54:4	sample 1	**1.83**	11.6	**1.89**	15.3
	sample 2	**0.93**	11	**0.96**	11.6
	sample 3	**37.41**	4.1	**37.39**	5.7
	sample 4	**175.39**	10.5	**146.61**	26.1
	sample 5	**0.65**	28.9	**0.66**	147.5
TG 54:3	sample 1	**4.78**	11.3	**4.91**	14.2
	sample 2	**0.44**	12	**0.46**	12.8
	sample 3	**18.69**	4.2	**18.72**	5.8
	sample 4	**248.23**	12.4	**225.33**	11.5
	sample 5	**0.6**	88.8	**0.29**	131

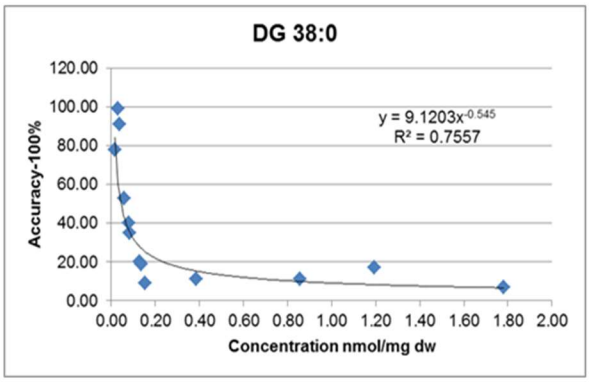
3.1.2 Limit of Quantification

Higher CV values were most likely related to concentrations close to limit of detection. Therefore, limit of quantification (LoQ) was determined functionally as described previously (124, 169). The LoQ was defined as concentration at which either the CV reached 20% or accuracy left the range of 80-120%. Non-endogenous DG and TG species were spiked at various concentrations and analyzed in fivefold. CV and accuracy were fitted as shown in Figure 3.4. The calculated LoQs were in the range of 0.01-0.2 nmol/mg dw for DG species and 0.01-0.3 nmol/mg dw for TG species. LoQs determined at CV of 20% were significantly lower compared to those determined by accuracy. Most of the LoQs derived from CVs were in the range of 0.01 to 0.02 nmol/mg dw which also matched the inter- and intra-day CVs listed in Table 3.2 and Table 3.3. This demonstrates a reproducible analysis below 0.1 nmol/mg dw. LoQs derived from accuracy analysis depend on accurate addition of low amounts of DG/TG species, which may be compromised by different factors including analyte absorption or inhomogeneity issues (see *3.1.4 Evaluation of Reproducibility Issues*). Except a poor curve fit as a factor, we could not find an explanation for the order of magnitude difference between the LoQs determined for different species. There seems to be neither a relation to species chain length nor to number of double bonds. LoQs for DG and TG appear to be similar. Based on these considerations, we applied 0.02 nmol/mg dw as LoD and 0.1 nmol/mg dw as LoQ for practical reasons (this is also substantiated by data from dilution integrity testing shown under *3.1.3 Recovery, Linearity and Dilution Integrity*).

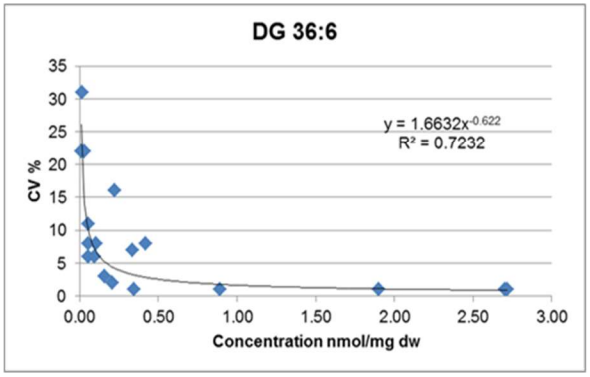
Validation of this method demonstrated its suitability for large scale studies including extraction, FIA-FTMS analysis, and data evaluation despite the higher variations observed for some samples.



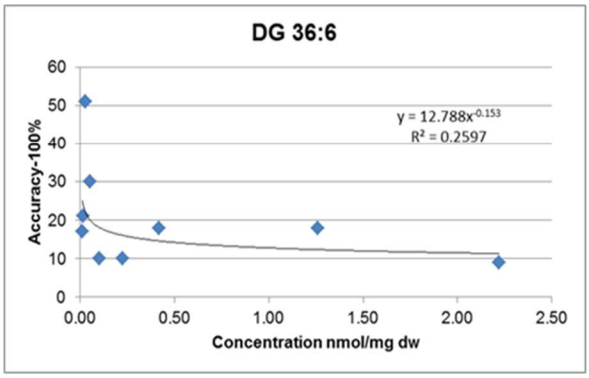
LoQ at CV = 20%: 0.02 nmol/mg dw



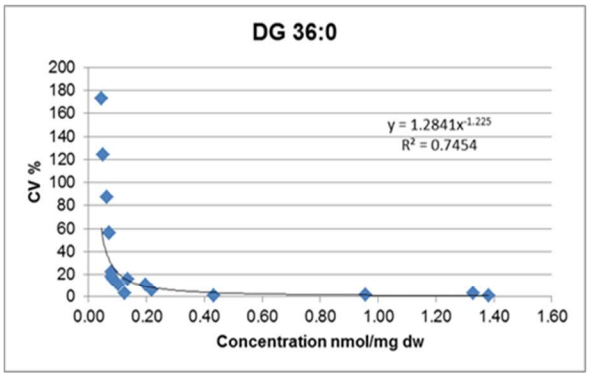
LoQ at trueness-100 = 20%: 0.2 nmol/mg dw



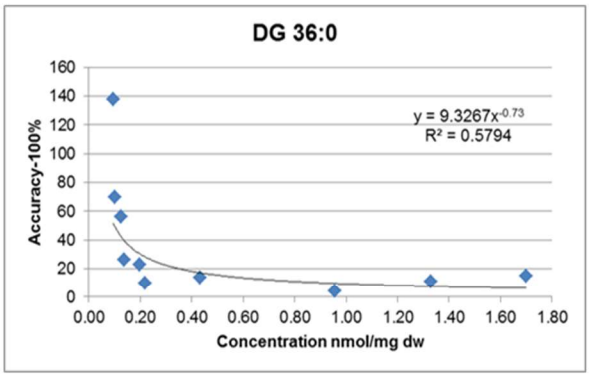
LoQ at CV = 20%: 0.02 nmol/mg dw



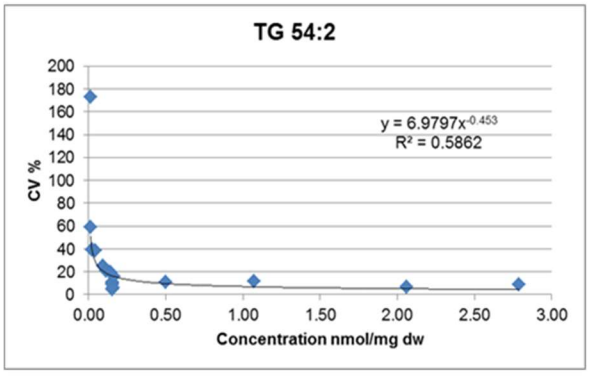
LoQ at trueness-100 = 20%: 0.05 nmol/mg dw



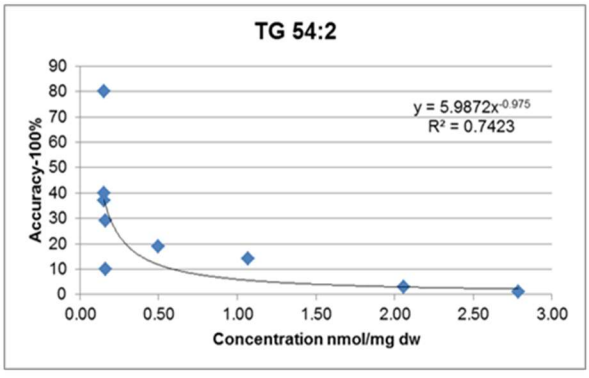
LoQ at CV = 20%: 0.1 nmol/mg dw



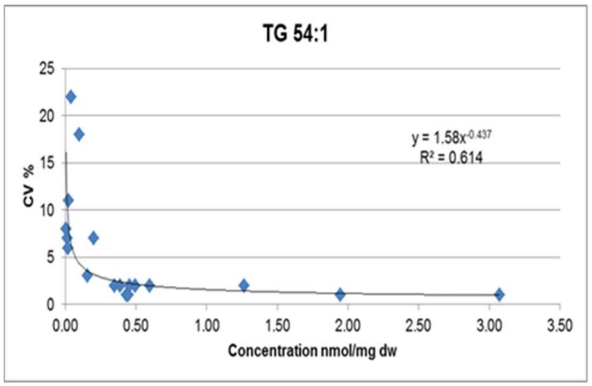
LoQ at trueness-100 = 20%: 0.3 nmol/mg dw

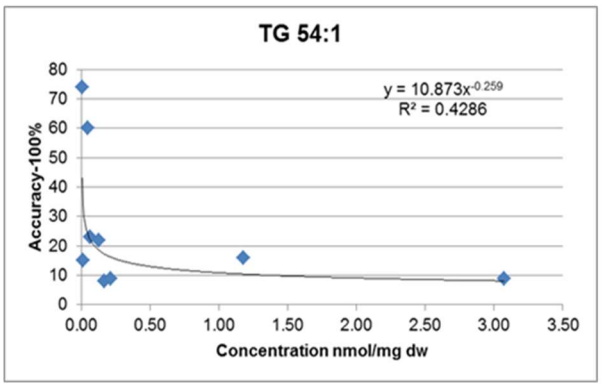


LoQ at CV = 20%: 0.1 nmol/mg dw



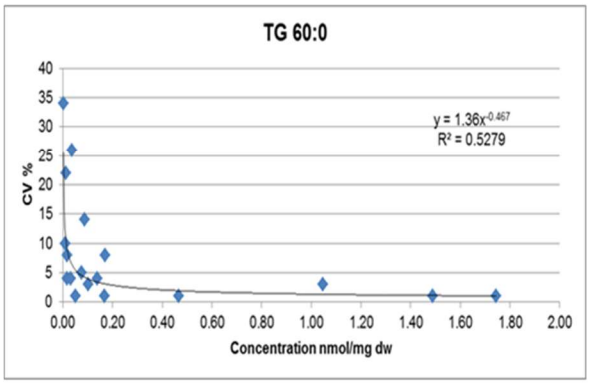
LoQ at trueness-100 = 20%: 0.3 nmol/mg dw

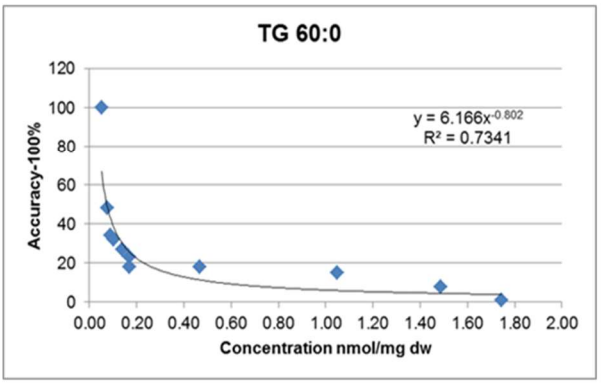




LoQ at CV = 20%: 0.01 nmol/mg dw

LoQ at trueness-100 = 20%: 0.09 nmol/mg dw





LoQ at CV = 20%: 0.01 nmol/mg dw

LoQ at trueness-100 = 20%: 0.2 nmol/mg dw

Figure 3.4: Calculation of LoQ for DG 38:0, DG 36:6, DG 36:0, TG 54:2, TG 54:1 and TG 60:0 from serial dilutions of different human fecal samples each analyzed in fivefold. Left panels illustrate the measured CVs plotted against the concentration of undiluted samples, respectively. Right panels show the absolute values of trueness-100 plotted against the concentration of undiluted samples, respectively. The results were fitted by a power function and concentrations were calculated at CV = 20% or at absolute values of trueness-100 = 20%.

3.1.3 Recovery, Linearity and Dilution Integrity

Recovery of DG and TG species was determined at two spike levels (Table 3.4). Most of the determined recoveries were within the expected range of 85 to 115%. However, considering the high complexity of fecal material as matrix, we think that recoveries between 75% and 135% are acceptable.

Table 3.4: Recovery data of DG and TG species in human feces. Concentrations were determined in triplicates.

Compound		Spiked concentration [nmol/mg dw]	Concentration ± standard deviation [nmol/mg dw]			Recovery [%]
	unspiked		n.d.	±	n.d.	
DG 36:6	Spike low	3.27	4.33	±	0.21	132.6
	Spike high	16.3	17.3	±	0.95	105.9
	unspiked		0.99	±	n.d.	
DG 36:4	Spike low	3.24	3.93	±	0.13	121.1
	Spike high	16.2	16.2	±	0.86	99.8
	unspiked		n.d.	±	n.d.	
DG 36:0	Spike low	3.20	2.72	±	0.09	84.8
	Spike high	16.0	12.1	±	0.42	75.3
	unspiked		n.d.	±	n.d.	
DG 38:0	Spike low	3.07	3.13	±	n.d.	102.0
	Spike high	15.3	14.8	±	0.47	96.8
	unspiked		0.13	±	n.d.	
TG 48:0	Spike low	2.48	2.04	±	0.12	82.1
	Spike high	12.4	10.4	±	0.44	84.3
	unspiked		n.d.	±	n.d.	
TG 54:6	Spike low	2.28	2.52	±	0.10	110.9
	Spike high	11.4	12.7	±	0.89	111.6
	unspiked		n.d.	±	n.d.	
TG 54:3	Spike low	2.26	2.04	±	n.d.	90.2
	Spike high	11.3	10.2	±	0.65	90.5
	unspiked		n.d.	±	n.d.	
TG 54:1	Spike low	2.25	2.32	±	n.d.	103.3
	Spike high	11.3	11.8	±	0.54	104.7

A major goal of high-resolution shotgun lipidomics approaches is an accurate quantification of lipid species. To further evaluate the dynamic range of the method the linearity of quantification was tested for several species not present in fecal samples. DG 36:6, DG 38:0, TG 54:2, and TG 54:1 were spiked at six different concentrations (Figure 3.5). All species revealed a good correlation of spiked and detected concentrations. A linear increase was observed with similar slopes for pure standards and matrix containing samples, which excludes significant effects of the matrix. However, species response seems to depend on structural features, as described for cholesteryl ester (164), and should be examined in detail in further studies. A linear range covering most of the tested samples was demonstrated up to 120 mg dw/mL and 90 mg dw/mL for DG and TG, respectively.

Moreover, dilution integrity was tested by quantification of gradually diluted stool samples (1.6 mg dw/mL to 0.02 mg dw/mL). Low (DG 32:0, TG 48:0), medium (DG 34:2, TG 52:2) and high (DG 36:3, TG 54:4) abundant species showed good correlation of expected and measured concentrations (Figure 3.6). The assay was linear at low (DG 32:0 and TG 48:0) and high concentrations (DG 36:3, TG 54:4) and matched the above described LoQ and LoD and linear range (up to 250 mg DG dw/mL and 150 mg TG dw/mL), respectively.
The concentration range of each synthetic standard tested lies within the concentration range of the fecal samples. Based on this result, we conclude that the FIA-FTMS method using shotgun lipidomics is applicable for quantitative analysis of fecal lipids.

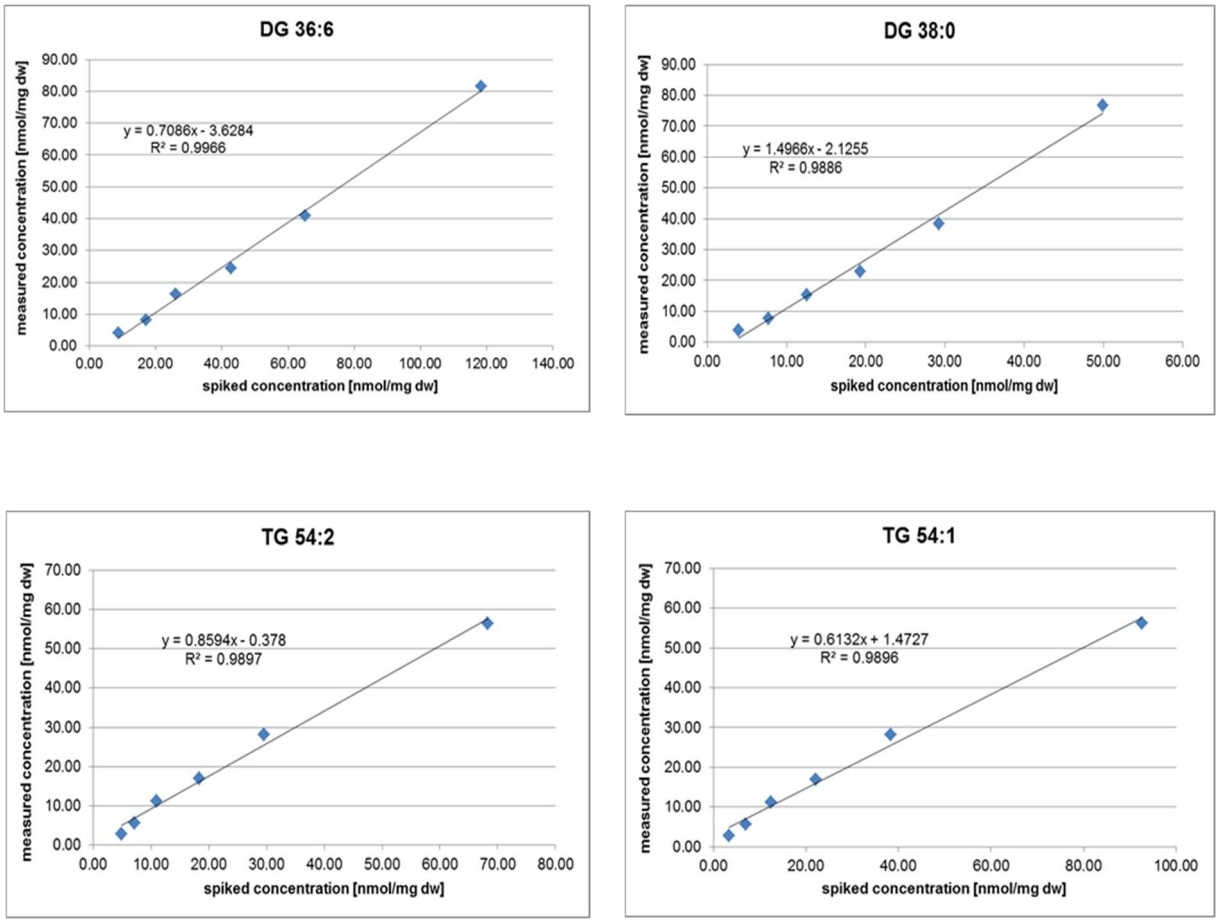


Figure 3.5: Linearity of DG and TG standards. Means (n=5) of the measured were plotted against the spiked concentrations.

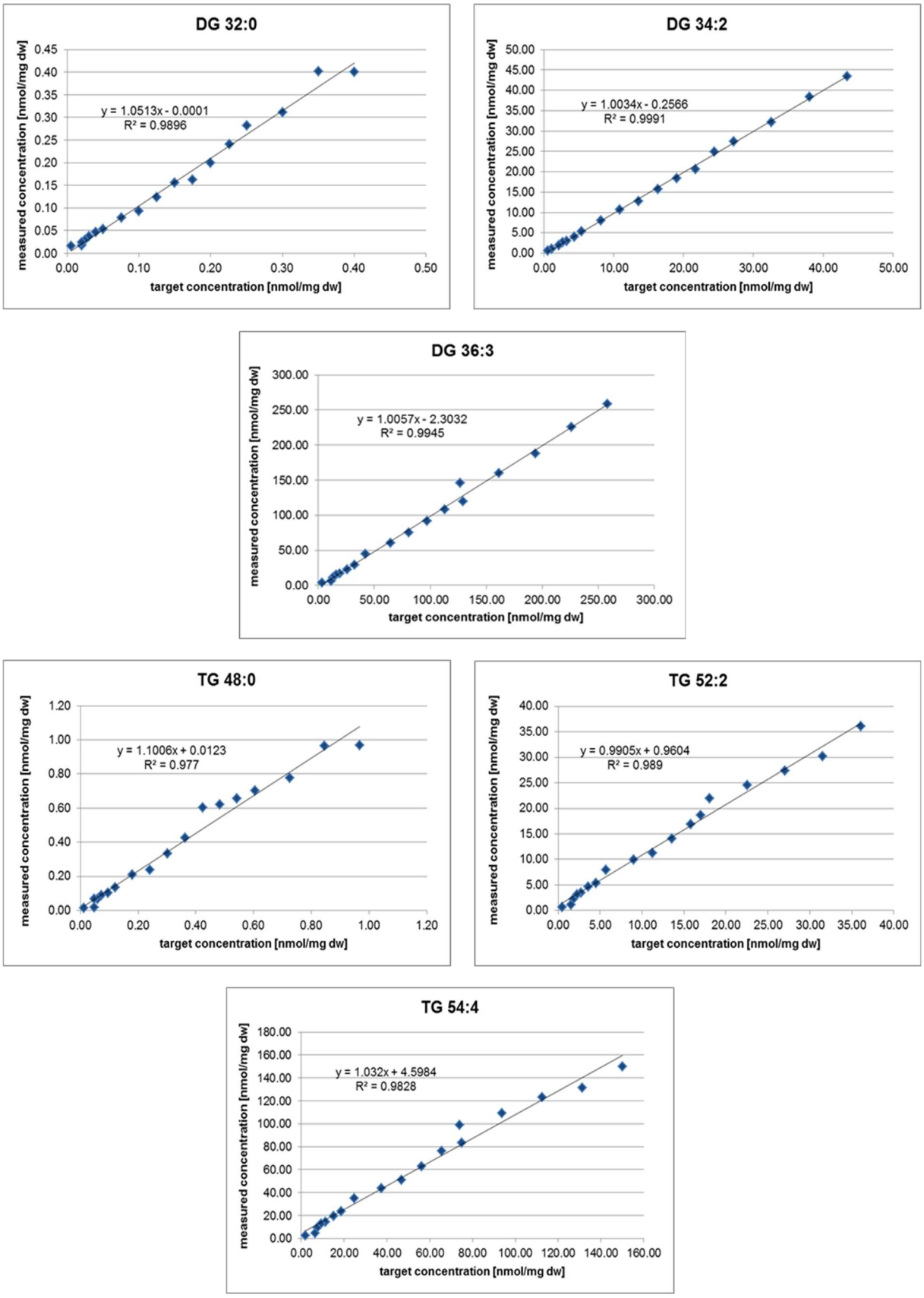


Figure 3.6: Dilution integrity of DG and TG species with low, medium, and high concentrations, respectively. Samples were analyzed in triplicates. The mean measured concentrations were plotted against the target concentrations.

3.1.4 Evaluation of Reproducibility Issues

As described above, very high variations were observed for some samples (about 10% of tested fecal samples). Therefore, various experiments were performed to evaluate the origin of irreproducibility. Despite thorough mechanical homogenization fecal samples are suspensions and a lack of homogeneity may cause variations.

3.1.4.1 Effect of Centrifugation

First, we tested whether centrifugation affected DG and TG concentrations. Five samples showing high variations were analyzed without centrifugation as well as after centrifugation (Table 3.5 and Table 3.6).

Table 3.5: Five different samples were analyzed without centrifugation as well as their supernatant and pellet after centrifugation. Each sample was analyzed in triplicates. Mean DG species concentrations and the fraction found in supernatant and pellet are displayed.

Diacylglycerol	sample	without centrifugation [nmol/mg dw]	supernatant [%]	pellet [%]
DG 34:3	sample 1	0.19	46.5	53.5
	sample 2	0.13	53.0	47.0
	sample 3	n.d.	53.1	46.9
	sample 4	0.13	57.9	42.1
	sample 5	0.06	41.7	58.3
DG 34:2	sample 1	2.97	47.9	52.1
	sample 2	1.62	48.3	51.7
	sample 3	0.56	46.4	53.6
	sample 4	1.15	53.4	46.6
	sample 5	1.16	44.2	55.8
DG 34:1	sample 1	0.78	48.0	52.0
	sample 2	6.73	48.3	51.7
	sample 3	2.07	45.6	54.4
	sample 4	1.48	53.4	46.6
	sample 5	0.20	46.6	53.4
DG 36:5	sample 1	0.20	47.8	52.2
	sample 2	0.10	50.3	49.7
	sample 3	n.d.	59.7	40.3
	sample 4	0.24	55.0	45.0
	sample 5	0.25	45.2	54.8
DG 36:4	sample 1	13.35	47.9	52.1
	sample 2	3.15	50.0	50.0
	sample 3	1.19	51.4	48.6
	sample 4	3.66	55.4	44.6
	sample 5	3.81	45.1	54.9
DG 36:3	sample 1	5.85	48.1	51.9
	sample 2	12.21	48.5	51.5
	sample 3	3.42	47.2	52.8
	sample 4	4.43	53.8	46.2
	sample 5	1.88	45.6	54.4
DG 36:2	sample 1	2.79	47.6	52.4
	sample 2	47.41	48.0	52.0
	sample 3	13.67	45.7	54.3
	sample 4	7.25	53.1	46.9
	sample 5	1.25	46.1	53.9

Table 3.6: Five different samples were analyzed without centrifugation as well as their supernatant and pellet after centrifugation. Each sample was analyzed in triplicates. Mean TG species concentrations and the fraction found in supernatant and pellet are displayed.

Triacylglycerol	sample	without centrifugation [nmol/mg dw]	supernatant [%]	pellet [%]
TG 52:4	sample 1	0.26	47.8	52.2
	sample 2	0.24	22.0	78.0
	sample 3	0.18	20.7	79.3
	sample 4	0.13	44.0	56.0
	sample 5	0.13	11.2	88.8
TG 52:3	sample 1	n.d.	45.8	54.2
	sample 2	0.75	27.1	72.9
	sample 3	0.73	11.7	88.3
	sample 4	0.22	38.4	61.6
	sample 5	n.d.	8.3	91.7
TG 52:2	sample 1	n.d.	40.5	59.5
	sample 2	2.65	25.7	74.3
	sample 3	2.68	9.7	90.3
	sample 4	0.63	37.2	62.8
	sample 5	n.d.	8.4	91.6
TG 54:7	sample 1	n.d.	44.1	55.9
	sample 2	n.d.	17.2	82.8
	sample 3	n.d.	15.4	84.6
	sample 4	n.d.	48.2	51.8
	sample 5	n.d.	11.8	88.2
TG 54:6	sample 1	0.46	47.8	52.2
	sample 2	0.39	17.1	82.9
	sample 3	0.21	21.9	78.1
	sample 4	0.17	44.3	55.7
	sample 5	0.15	8.7	91.3
TG 54:5	sample 1	0.31	47.8	52.2
	sample 2	1.12	30.2	69.8
	sample 3	0.92	14.2	85.8
	sample 4	0.42	38.7	61.3
	sample 5	n.d.	9.1	90.9
TG 54:4	sample 1	0.20	47.3	52.7
	sample 2	4.29	27.7	72.3
	sample 3	3.73	10.6	89.4
	sample 4	1.06	35.7	64.3
	sample 5	n.d.	9.1	90.9
TG 54:3	sample 1	n.d.	47.6	52.4
	sample 2	14.66	27.5	72.5
	sample 3	13.59	8.7	91.3
	sample 4	2.99	38.2	61.8
	sample 5	n.d.	12.3	87.7

Whereas DG species were detected in both pellet and supernatant, TG species were found in three of the samples enriched in the pellet (Figure 3.7). However, the DG/TG species profiles of supernatant and pellet closely resembled each other (Table 3.7 and Table 3.8), suggesting that centrifugation does not separate a specific pool of these lipid classes. However, due to the substantial amount of DG and TG in sample pellets, analysis of sample supernatants does not permit accurate quantification.

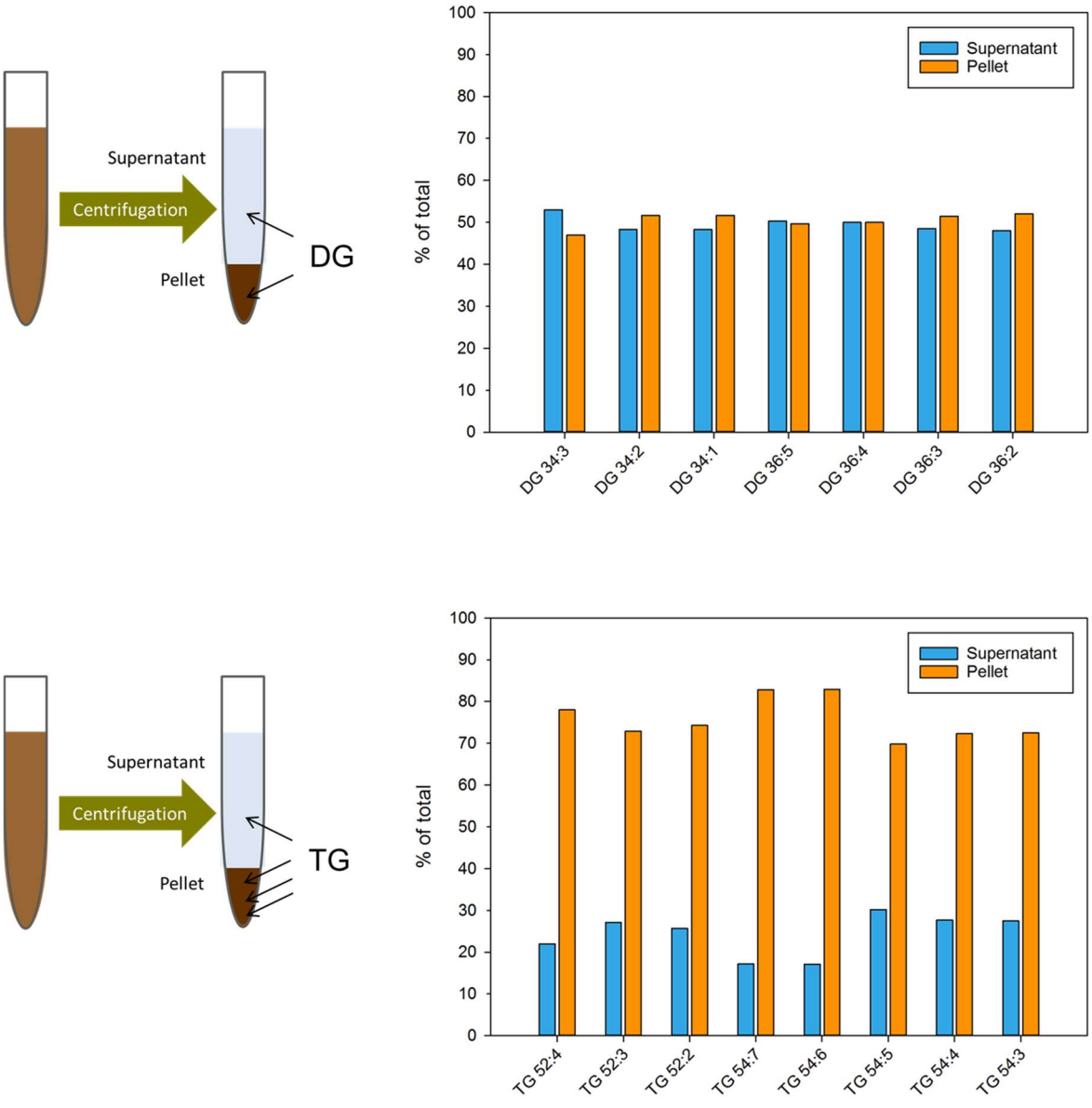


Figure 3.7: Five different samples were analyzed without centrifugation as well as their supernatant and pellet after centrifugation. Each sample was analyzed in triplicates. Fractions found of either DG or TG species in supernatant and pellet are displayed, respectively. The panels show the values of only one sample which is characteristic for all measured samples.

Table 3.7: Species profile in % total DG of the data listed in Table 3.5.

Species	sample 1			sample 2			sample 3			sample 4			sample 5		
	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]
DG 34:3	0.7	0.4	0.5	0.2	0.2	0.1	0.3	0.3	0.2	0.7	0.6	0.5	0.7	0.6	0.7
DG 34:2	11.2	11.5	11.6	2.2	2.2	2.2	2.5	2.6	2.6	5.9	6.0	6.0	12.7	12.5	13.1
DG 34:1	2.9	2.0	2.0	9.1	9.2	9.2	9.3	9.3	9.5	7.6	7.3	7.5	2.2	2.2	2.1
DG 36:5	0.7	0.6	0.6	0.1	0.1	0.1	0.2	0.2	0.1	1.2	1.2	1.1	2.7	2.7	2.7
DG 36:4	50.1	49.0	49.6	4.3	4.2	3.9	5.4	5.3	4.3	18.8	18.3	17.2	41.5	41.5	42.0
DG 36:3	22.0	23.2	23.3	16.6	16.6	16.4	15.5	16.0	15.4	22.8	22.9	23.0	20.5	20.2	20.1
DG 36:2	10.5	10.7	11.0	64.4	64.6	65.0	61.8	61.5	62.8	37.3	38.1	39.2	13.7	13.8	13.5

Table 3.8: Species profile in % total TG of the data listed in Table 3.6.

Species	sample 1			sample 2			sample 3			sample 4			sample 5		
	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]	without centrifug [%]	supernatant [%]	pellet [%]
TG 52:4	18.0	19.3	19.0	1.0	1.0	1.3	0.8	1.6	0.7	2.3	3.0	2.4	24.0	24.5	20.4
TG 52:3	5.7	5.3	5.7	3.1	3.2	3.2	3.3	3.8	3.0	4.0	4.3	4.2	6.9	7.0	8.1
TG 52:2	3.0	2.3	3.0	11.0	10.7	11.5	12.2	12.0	11.9	11.2	11.4	11.8	4.0	3.1	3.6
TG 54:7	0.9	0.9	1.0	0.1	0.1	0.1	0.0	0.1	0.0	0.3	0.4	0.3	3.8	3.9	3.1
TG 54:6	31.7	32.3	31.7	1.6	1.5	2.8	0.9	1.7	0.7	3.0	3.7	2.8	28.1	26.9	29.7
TG 54:5	21.8	21.6	21.2	4.7	4.9	4.2	4.2	5.5	3.5	7.5	8.3	8.1	17.2	17.8	18.9
TG 54:4	14.1	13.8	13.8	17.8	18.2	17.6	16.9	18.1	16.3	18.7	17.9	19.8	10.9	11.3	12.0
TG 54:3	4.9	4.5	4.5	60.8	60.5	59.4	61.6	57.3	63.9	53.0	51.0	50.6	5.1	5.5	4.1

3.1.4.2 Effect of Bead Beating

In order to improve the homogeneity of the fecal samples, a number of different experiments were carried out. After homogenization using the gentleMACS™ dissociator (as described under *2.5.1 Feces Homogenization*) different samples were tested with an additional homogenization step using the Precellys® homogenizer (data shown in Table 3.9 and Table 3.10). For this experiment, a sample showing high variation in preliminary tests was homogenized again and then divided into four different Precellys ® tubes and measured in fivefold.

Table 3.9: One sample was divided into four tubes and analyzed either with or without Precellys® after homogenization. Each sample was analyzed in fivefold. Mean DG species concentrations and the coefficient of variation (CV) are displayed.

		without Precellys®		**Precellys®**	
Diacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
DG 34:2	sample I	**0.48**	12.9	**0.51**	7.8
	sample II			**0.53**	11.3
	sample III			**0.48**	8.0
	sample IV			**0.53**	7.8
DG 34:1	sample I	**0.19**	9.8	**0.17**	6.1
	sample II			**0.22**	9.9
	sample III			**0.19**	9.4
	sample IV			**0.19**	8.8
DG 36:4	sample I	**2.30**	7.1	**2.46**	5.2
	sample II			**2.49**	10.9
	sample III			**2.30**	5.7
	sample IV			**2.50**	8.4
DG 36:3	sample I	**2.02**	11.2	**2.17**	8.5
	sample II			**2.30**	11.8
	sample III			**2.16**	5.3
	sample IV			**2.31**	8.0
DG 36:2	sample I	**0.69**	9.6	**0.73**	5.9
	sample II			**0.80**	10.4
	sample III			**0.73**	7.7
	sample IV			**0.78**	4.4

Table 3.10: One sample was divided into four tubes and analyzed either with or without Precellys® after homogenization. Each sample was analyzed in fivefold. Mean TG species concentrations and the coefficient of variation (CV) are displayed.

		without Precellys®		**Precellys®**	
Triacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
TG 50:2	sample I	**0.34**	16.3	**0.26**	10.4
	sample II			**0.3**	11.4
	sample III			**0.24**	10.5
	sample IV			**0.27**	9.7
TG 52:4	sample I	**3.37**	14	**2.76**	9.9
	sample II			**3.06**	11.6
	sample III			**2.61**	11.3
	sample IV			**2.81**	10.9
TG 52:3	sample I	**2.19**	12.2	**1.74**	12.2
	sample II			**1.84**	12.2
	sample III			**1.64**	9.7
	sample IV			**1.64**	11.9
TG 52:2	sample I	**0.79**	15.2	**0.58**	13
	sample II			**0.7**	11.2
	sample III			**0.56**	8.9
	sample IV			**0.59**	6.4
TG 54:6	sample I	**9.69**	8.2	**8.06**	9
	sample II			**8.86**	13
	sample III			**7.79**	11.1
	sample IV			**7.83**	13.6
TG 54:5	sample I	**12.62**	5.9	**10.64**	9.4
	sample II			**11.64**	13.6
	sample III			**10.24**	10.5
	sample IV			**10.21**	12.9
TG 54:4	sample I	**6.39**	8.6	**5.25**	11.5
	sample II			**5.9**	13.2
	sample III			**4.99**	9.2
	sample IV			**5.14**	9.5
TG 54:3	sample I	**2.25**	8.3	**1.85**	13.3
	sample II			**2.04**	13
	sample III			**1.74**	8.3
	sample IV			**1.76**	10.3

Samples showed CVs < 17% for all DG and TG species. Whereas CVs for TG decreased, similar CVs were observed for DG species. In summary, bead-beating did not result in significantly higher reproducibility.

Furthermore, a decrease of TG species was observed which may be related to adsorption to the beads or lipolysis during this homogenization step.

3.1.4.3 Effect of Sample Concentration and Volume

In a next step, we asked whether the amount of fecal material used for homogenization could improve reproducibility. Therefore, two approaches were followed. In the first step, fecal homogenates were adjusted to different concentrations: 0.5 mg dw/mL, 1.0 mg dw/mL, and 2.0 mg dw/mL. An amount corresponding to 200 µg dry weight, i.e. 100 µL, 200 µL, or 400 µL was used for extraction, thus sample amount was equal in all cases. For this experiment, two different raw fecal samples were homogenized and analyzed in fivefold. The data are shown in Table 3.11 and Table 3.12. Samples with a lower concentration and higher sample volume did not exhibit lower CVs. On the contrary, the CVs of triacylglycerol species were significantly higher (CV > 15%) in some cases compared to 2.0 mg dw/mL, with up to 52% for sample 1 and a dry weight of 0.5 mg dw/mL (TG 54:6).

Both samples showed an increase in concentration of TG species using a higher sample concentration which could be related to particle aggregation at higher sample concentration. Aggregates (containing high amounts of TG) may result in an increased proportion of TG.

Table 3.11: Different sample concentrations (0.5 mg dw/mL, 1.0 mg dw/mL, and 2.0 mg dw/mL) of raw human fecal material were used for analysis of two different samples. An amount of either 100 µL, 200 µL or 400 µL was used for extraction. Each sample was analyzed in fivefold. Mean DG species concentrations and the coefficient of variation (CV) are displayed.

		Sample concentration					
		0.5 mg dw/mL		1.0 mg dw/mL		2.0 mg dw/mL	
Diacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
DG 34:3	sample I	**0.20**	13.0	**0.20**	6.9	**0.20**	8.7
	sample II	**0.42**	2.0	**0.60**	9.5	**0.42**	8.3
DG 34:2	sample I	**3.63**	2.3	**3.59**	7.0	**3.65**	7.9
	sample II	**5.04**	2.6	**7.35**	9.9	**5.42**	8.1
DG 34:1	sample I	**1.87**	6.4	**1.88**	7.2	**1.92**	10.2
	sample II	**3.87**	5.8	**5.52**	10.1	**4.39**	7.3
DG 36:5	sample I	**0.48**	7.1	**0.48**	6.0	**0.46**	7.4
	sample II	**n.d.**		**n.d.**		**n.d.**	
DG 36:4	sample I	**10.77**	6.0	**10.82**	7.0	**10.70**	7.4
	sample II	**9.03**	2.5	**13.33**	10.3	**9.64**	7.2
DG 36:3	sample I	**10.03**	6.0	**10.08**	6.0	**10.12**	7.6
	sample II	**29.81**	3.6	**43.55**	10.5	**31.88**	6.3
DG 36:2	sample I	**5.38**	5.2	**5.44**	7.01	**5.62**	9.6
	sample II	**37.04**	4.5	**52.92**	10.5	**39.46**	5.6

Table 3.12: Different sample concentrations (0.5 mg dw/mL, 1.0 mg dw/mL, and 2.0 mg dw/mL) of raw human fecal material were used for analysis of two different samples. An amount of either 100 µL, 200 µL or 400 µL was used for extraction. Each sample was analyzed in fivefold. Mean TG species concentrations and the coefficient of variation (CV) are displayed.

		Sample concentration					
		0.5 mg dw/mL		1.0 mg dw/mL		2.0 mg dw/mL	
Triacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
TG 50:2	sample I	**n.d.**		**n.d.**		**n.d.**	
	sample II	**1.54**	9.4	**2.13**	11.7	**2.61**	7.8
TG 50:1	sample I	**n.d.**		**n.d.**		**n.d.**	
	sample II	**0.45**	9.5	**0.59**	11.8	**0.73**	9.9
TG 52:5	sample I	**n.d.**		**n.d.**		**n.d.**	
	sample II	**0.87**	7.5	**1.21**	11.9	**1.47**	7.5
TG 52:4	sample I	**0.43**	49.6	**0.36**	17.3	**0.52**	6.0
	sample II	**9.03**	9.2	**12.51**	10.0	**15.38**	8.8
TG 52:3	sample I	**0.27**	27.8	**0.33**	35.3	**0.46**	2.2
	sample II	**29.28**	9.6	**39.88**	9.8	**47.99**	7.8
TG 52:2	sample I	**0.18**	33.8	**0.20**	42.2	**0.27**	7.0
	sample II	**20.65**	9.6	**27.55**	10.0	**32.99**	7.4
TG 54:6	sample I	**0.49**	51.8	**0.47**	23.8	**0.70**	13.6
	sample II	**9.14**	9.2	**12.68**	9.6	**15.55**	9.0
TG 54:5	sample I	**0.62**	49.8	**0.69**	35.3	**0.97**	6.1
	sample II	**48.01**	8.9	**66.36**	9.8	**80.12**	8.0
TG 54:4	sample I	**0.56**	47.8	**0.69**	45.5	**0.92**	6.4
	sample II	**81.43**	9.8	**111.71**	9.9	**135.24**	8.6
TG 54:3	sample I	**0.36**	43.3	**0.50**	6.9	**0.67**	24.3
	sample II	**114.93**	10.0	**158.09**	9.8	**192.43**	9.0

In the second approach, different volumes (100 µL and 400 µL) of the homogenized fecal samples were subjected to lipid extraction (Table 3.13 and Table 3.14). Mass spectrometric analysis was performed at the same concentration. In this experiment samples with 2.0 mg dw/mL from the previous tests were used. The application of higher sample volumes showed some decrease of CV, especially for TG species and samples with a high fraction of TG in the pellet. Considering that variation is mainly due to sample inhomogeneity, using a higher sample volume could explain lower variations.

Table 3.13: Two different volumes of homogenized fecal material (2.0 mg dw/mL) were used for analysis of two different samples. Each sample was analyzed in fivefold. Mean DG species concentrations and the coefficient of variation (CV) are displayed.

		Volume			
		100 µL		400 µL	
Diacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
DG 34:3	sample I	**0.18**	6.8	**0.20**	6.7
	sample II	**0.43**	5.8	**0.41**	2.8
DG 34:2	sample I	**3.34**	6.3	**3.49**	3.8
	sample II	**5.50**	3.5	**5.25**	3.4
DG 34:1	sample I	**1.83**	6.5	**1.79**	4.3
	sample II	**4.32**	4.8	**4.19**	1.3
DG 36:5	sample I	**0.42**	5.7	**0.42**	5.6
	sample II	**n.d.**		**n.d.**	
DG 36:4	sample I	**9.63**	5.4	**10.04**	5.2
	sample II	**9.89**	4.1	**9.24**	4.9
DG 36:3	sample I	**9.33**	9.5	**9.56**	4.6
	sample II	**33.06**	4.3	**30.84**	4.3
DG 36:2	sample I	**5.44**	11.2	**5.14**	4.2
	sample II	**40.56**	4.7	**38.29**	2.7

Table 3.14: Two different volumes of homogenized fecal material (2.0 mg dw/mL) were used for analysis of two different samples. Each sample was analyzed in fivefold. Mean TG species concentrations and the coefficient of variation (CV) are displayed.

		Volume			
		100 µL		400 µL	
Triacylglycerol	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
TG 50:2	sample I	**n.d.**		**n.d.**	
	sample II	**2.13**	7.7	**2.05**	3.4
TG 50:1	sample I	**n.d.**		**n.d.**	
	sample II	**0.60**	6.0	**0.55**	7.6
TG 52:5	sample I	**n.d.**		**n.d.**	
	sample II	**1.17**	6.5	**1.10**	4.3
TG 52:4	sample I	**0.39**	8.9	**0.41**	5.0
	sample II	**12.21**	7.4	**11.68**	2.8
TG 52:3	sample I	**0.29**	13.2	**0.30**	8.0
	sample II	**37.98**	7.8	**36.07**	3.4
TG 52:2	sample I	**0.17**	21.8	**0.17**	6.2
	sample II	**26.57**	7.8	**24.97**	3.4
TG 54:6	sample I	**0.48**	7.9	**0.53**	10.0
	sample II	**12.02**	7.3	**11.61**	3.3
TG 54:5	sample I	**0.61**	10.0	**0.67**	9.7
	sample II	**63.32**	8.0	**60.25**	3.1
TG 54:4	sample I	**0.55**	16.0	**0.60**	8.2
	sample II	**107.72**	7.6	**102.29**	2.7
TG 54:3	sample I	**0.35**	26.9	**0.37**	10.0
	sample II	**156.97**	7.1	**148.47**	2.4

3.1.4.4 Effect of Lipid Extraction

Besides sample homogeneity, the effect of lipid extraction on DG and TG concentrations was also tested. One of these experiments involved variation of incubation time after addition of both internal standard and Bligh and Dyer mixture to the homogenized fecal sample. The results are shown in Table 3.15 - Table 3.17. For both DG and TG species, incubation time seemed to play a minor role with a plateau observed after 10 min incubation time. The detected concentrations varied only slightly after 10 min, except for sample 2 for TG species. Species profiles showed no variation between the different incubation times, except for TG species profile for sample 2. An incubation time of 60 min, as regularly used, seemed to be sufficient, and is therefore still used in the following experiments. Combination of different incubation times with additional sonication up to 3 h during extraction is shown in Table 3.18. Both incubation times and simultaneous treatment with sonication did not result in significant improvement in variation.

Table 3.15: Four different samples were analyzed. Incubation time before phase separation varied from 0 minutes to 24 hours. Each sample was analyzed only once. Sum concentrations of DG and TG species for each sample are displayed.

	Diacylglycerol				Triacylglycerol			
incubation time	sample 1 [nmol/mg dw]	sample 2 [nmol/mg dw]	sample 3 [nmol/mg dw]	sample 4 [nmol/mg dw]	sample 1 [nmol/mg dw]	sample 2 [nmol/mg dw]	sample 3 [nmol/mg dw]	sample 4 [nmol/mg dw]
0 minutes	34.13	28.13	37.08	14.63	7.62	8.35	11.64	49.38
10 minutes	32.53	31.8	31.90	19.35	8.72	3.30	8.67	55.63
20 minutes	32.62	32.71	32.67	18.74	9.87	12.22	8.83	47.26
30 minutes	33.16	32.99	30.20	19.47	7.33	3.81	7.92	52.98
60 minutes	32.32	32.88	29.90	19.22	7.54	5.19	7.21	48.04
180 minutes	30.95	29.98	28.40	19.35	7.48	3.68	6.68	49.76
24 hours	30.19	30.27	27.01	18.05	7.58	5.09	6.75	49.11

Table 3.16: Species profile in % total DG of the data listed in Table 3.15.

	sample 1							sample 2						
Species	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]
DG 34:3	0.6	0.6	0.6	0.6	0.5	0.6	0.6	1.3	1.4	1.3	1.3	1.3	1.3	1.4
DG 34:2	10.4	10.1	10.1	10.1	9.9	10.1	10.3	4.9	5.0	4.9	4.9	4.9	4.9	4.9
DG 34:1	6.5	6.1	6.2	6.3	6.3	6.1	5.9	2.9	2.9	3.0	3.0	3.0	2.9	3.2
DG 36:5	0.9	1.0	1.1	1.0	1.0	1.1	1.2	30.0	29.6	30.0	30.4	30.3	30.1	29.3
DG 36:4	27.0	28.3	28.4	28.3	28.1	28.5	28.3	42.6	42.7	42.4	41.8	42.0	42.3	42.7
DG 36:3	33.4	33.8	33.2	32.6	32.8	32.9	33.2	11.3	11.5	11.6	11.8	11.7	11.7	11.5
DG 36:2	21.2	20.1	20.6	21.2	21.3	20.8	20.5	7.0	6.9	6.8	6.9	6.9	6.8	7.0

	sample 3							sample 4						
Species	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]
DG 34:3	0.6	0.6	0.6	0.6	0.6	0.6	0.6	1.2	1.1	1.0	1.0	1.0	1.0	1.1
DG 34:2	10.0	10.1	10.2	9.9	10.2	10.1	10.0	14.1	14.3	13.8	13.8	13.6	14.2	14.0
DG 34:1	6.2	6.0	6.1	6.4	6.3	5.8	6.4	8.0	8.0	8.4	8.4	8.5	8.1	8.2
DG 36:5	1.1	1.1	1.1	1.0	1.0	1.2	1.1	1.7	1.7	1.7	1.8	1.8	1.8	1.8
DG 36:4	28.0	28.2	28.2	28.0	28.2	28.6	27.8	30.1	29.4	28.0	27.9	28.3	29.6	29.3
DG 36:3	33.3	34.0	33.1	32.8	32.6	33.4	32.9	25.7	25.6	26.9	27.2	27.0	25.5	25.9
DG 36:2	20.8	20.0	20.7	21.4	21.2	20.3	21.1	19.3	19.9	20.1	20.0	19.9	19.7	19.7

Table 3.17: Species profile in % total TG of the data listed inTable 3.15.

	sample 1							sample 2						
Species	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]
TG 50:2	1.9	1.8	1.7	1.8	1.8	1.8	1.7	0.4	0.8	0.3	0.7	0.6	0.8	0.9
TG 50:1	1.2	1.2	1.2	1.1	1.2	1.2	1.2	0.3	0.9	0.3	0.8	0.6	0.9	0.7
TG 52:5	0.3	0.4	0.4	0.4	0.4	0.4	0.4	3.7	3.9	3.7	4.0	4.5	2.8	3.5
TG 52:4	8.8	8.0	7.9	8.4	8.5	8.1	8.2	5.7	6.6	5.2	6.3	6.0	5.6	7.2
TG 52:3	10.7	10.7	10.9	10.5	10.4	10.3	10.6	1.3	2.0	1.3	2.1	1.5	4.4	3.6
TG 52:2	7.2	7.1	7.3	6.8	6.6	6.9	6.9	0.7	1.8	0.7	1.6	1.1	3.1	2.4
TG 54:7	0.9	0.9	0.8	1.0	1.0	1.0	1.0	33.3	31.6	35.0	30.9	35.8	22.9	22.1
TG 54:6	12.0	11.6	11.7	12.8	12.7	12.1	12.4	36.7	31.8	35.8	32.0	32.0	24.8	28.8
TG 54:5	20.8	21.2	21.4	21.7	21.7	21.2	21.4	12.3	11.4	12.0	11.8	11.0	13.2	14.1
TG 54:4	22.1	22.9	22.9	22.0	22.2	22.5	22.3	3.9	5.2	4.2	5.8	3.9	10.6	8.5
TG 54:3	14.0	14.3	14.1	13.5	13.5	14.6	13.8	1.8	4.1	1.6	3.9	2.9	10.9	8.0

	sample 3							sample 4						
Species	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]	0 minutes [%]	10 minutes [%]	20 minutes [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	24 hours [%]
TG 50:2	1.5	1.7	1.8	2.0	1.7	1.7	1.8	2.6	2.5	2.6	2.6	2.6	2.6	2.7
TG 50:1	1.0	1.0	1.2	1.2	1.1	1.2	1.2	1.1	1.0	1.1	1.1	1.1	1.1	1.1
TG 52:5	0.3	0.4	0.4	0.4	0.4	0.4	0.4	1.1	1.1	1.1	1.1	1.1	1.1	1.1
TG 52:4	7.8	8.2	8.5	8.6	8.3	8.3	8.2	12.0	12.2	12.0	12.1	12.1	12.1	12.1
TG 52:3	10.2	10.2	10.7	11.0	10.3	10.4	10.3	9.3	9.2	9.4	9.5	9.3	9.2	9.1
TG 52:2	6.3	6.0	6.7	6.7	6.6	6.9	6.7	5.4	5.2	5.6	5.6	5.6	5.5	5.9
TG 54:7	0.8	1.0	0.9	0.9	1.0	1.0	1.0	1.5	1.6	1.5	1.5	1.5	1.5	1.6
TG 54:6	12.8	13.2	12.7	12.4	12.5	12.4	12.4	18.0	18.4	17.7	17.7	17.7	17.7	17.4
TG 54:5	22.7	22.4	21.9	21.6	21.6	21.6	21.5	20.3	20.4	20.2	20.1	20.1	20.4	19.7
TG 54:4	23.0	22.8	22.0	21.9	22.7	22.2	22.6	17.5	17.2	17.4	17.4	17.4	17.4	17.4
TG 54:3	13.6	13.0	13.2	13.2	13.8	13.9	14.0	11.4	11.1	11.3	11.3	11.5	11.5	12.0

Table 3.18: Calculation of CV for DG and TG species either with or without ultrasonication during extraction. Incubation times varied from 10 to 180 minutes. Each sample (2.0 mg dw/mL) was analyzed in three replicates.

		without ultrasonication				**ultrasonication**			
		Diacylglycerol		Triacylglycerol		Diacylglycerol		Triacylglycerol	
	sample	Mean (n=3) [nmol/mg dw]	CV [%]	Mean (n=3) [nmol/mg dw]	CV [%]	Mean (n=3) [nmol/mg dw]	CV [%]	Mean (n=3) [nmol/mg dw]	CV [%]
10 minutes	sample I	**4.08**	8.3	**0.13**	11.7	**4.14**	9.9	**0.22**	89.4
	sample II	**48.69**	10.6	**4.74**	15.7	**46.91**	2.3	**4.87**	13.1
	sample III	**9.08**	6.9	**1.33**	19.8	**8.86**	4.2	**1.25**	11
30 minutes	sample I	**4.22**	4.6	**0.13**	10.2	**4.52**	1.1	**0.2**	51.8
	sample II	**50.73**	6.8	**4.99**	2.1	**46.93**	4.3	**5.06**	0.6
	sample III	**9.29**	5.7	**1.33**	10.5	**9.76**	4.1	**1.35**	13.8
60 minutes	sample I	**4.35**	1.9	**0.12**	5.2	**4.39**	6.3	**0.12**	1.8
	sample II	**48.85**	4.2	**5.04**	16.3	**50.15**	3.6	**5**	3
	sample III	**8.89**	3.2	**1.2**	5.2	**9.88**	3.1	**1.53**	33
120 minutes	sample I	**4.39**	2.6	**0.18**	55.9	**4.34**	3.2	**0.12**	3.8
	sample II	**49.79**	10.8	**4.67**	7.1	**47.36**	7.2	**4.65**	7.6
	sample III	**8.89**	1.5	**1.16**	2.9	**9.42**	4	**1.18**	2.8
180 minutes	sample I	**4.19**	1.7	**0.12**	5	**6.97**	64	**0.59**	136
	sample II	**48.89**	3.6	**6.18**	34.6	**49.91**	5.7	**4.55**	4.7
	sample III	**9.15**	8.7	**1.11**	5.2	**10.59**	6.4	**1.29**	17.2

3.1.4.5 Effect of Stool Grade

In a next step, we asked whether these inhomogeneity issues could be related to the consistency of the fecal material. Therefore, we selected, if available, three samples for each stool grade (according to Bristol Stool Chart (170); with grade 1 representing hard and grade 7 watery consistency) from a study on fibers and polyunsaturated fatty acids intervention (171). The samples were measured in five replicates (Table 3.19). Samples with grades 3 to 5 showed CVs ≤ 10%. However, in samples with lower grades (1 to 2) we could not see a clear trend for higher CVs, which may have been expected for more solid consistency.

Table 3.19: DG and TG concentrations and their coefficient of variation (n=5) related to stool grading.

		Diacylglycerol		Triacylglycerol	
	sample	Mean (n=5) [nmol/mg dw]	CV [%]	Mean (n=5) [nmol/mg dw]	CV [%]
Grade 1	sample a	**46.11**	6.6	**2.31**	11.6
	sample b	**15.71**	7.6	**14.25**	6.1
	sample c	**54.27**	6.4	**8.55**	10.7
Grade 2	sample d	**78.73**	7.1	**10.69**	10.9
	sample e	**38.65**	26.3	**82.10**	27.6
	sample f	**11.62**	2.1	**1.11**	20.8
Grade 3	sample g	**93.86**	9.2	**38.27**	10.2
	sample h	**27.30**	6.0	**2.65**	7.5
	sample i	**29.44**	6.5	**39.34**	5.1
Grade 4	sample j	**52.35**	3.0	**5.15**	7.9
	sample k	**14.30**	5.8	**1.87**	6.5
	sample l	**21.41**	2.7	**2.15**	2.5
Grade 5	sample m	**66.36**	6.4	**15.67**	6.0
	sample n	**25.91**	8.9	**19.96**	8.0
	sample o	**94.64**	4.2	**11.83**	5.5
Grade 6	sample p	**65.84**	4.8	**10.28**	5.6
	sample q	**44.92**	3.8	**21.40**	5.2
	sample r	**49.10**	17.0	**39.42**	15.2
Grade 7	sample s	**46.94**	22.2	**1.74**	18.6

3.1.4.6 Stability of Sample Extracts

In addition to homogenization and extraction experiments, the stability of samples after the extraction procedure was examined. Therefore, samples were subjected to lipid extraction and immediately measured on the Q Exactive Orbitrap. Subsequently, the samples were stored at different temperatures (-80°C, -20°C, 4°C, and RT) for one week and measured again. For both DG and TG species the analyzed concentrations hardly changed. The samples therefore showed good stability and could be stored at different temperatures for at least one week. Data are shown in Table 3.20.

Table 3.20: Concentrations of DG and TG species depending on storage at different temperatures for one week. Five different samples were measured. Each sample was analyzed only once. Samples were measured directly after lipid extraction (Reference) and again after one week of storage under different conditions. Sum concentrations of DG and TG species for each sample are displayed.

sample	temperatures	DG sum [nmol/mg dw]	TG sum [nmol/mg dw]
sample I	Reference	20.23	22.15
	-80°C	20.95	25.72
	-20°C	20.07	23.92
	4°C	19.03	21.92
	RT	19.18	21.00
sample II	Reference	3.34	1.13
	-80°C	3.07	1.19
	-20°C	3.19	1.10
	4°C	3.25	1.10
	RT	3.31	1.12
sample III	Reference	3.37	0.66
	-80°C	2.97	0.56
	-20°C	3.32	1.34
	4°C	3.27	0.34
	RT	3.49	0.36
sample IV	Reference	7.58	1.42
	-80°C	6.78	1.38
	-20°C	7.47	1.54
	4°C	7.18	1.27
	RT	9.19	1.58
sample V	Reference	0.75	0.22
	-80°C	0.78	0.23
	-20°C	0.74	0.22
	4°C	0.72	0.21
	RT	0.73	0.30

3.1.4.7 Summary – Homogenization and Extraction

Validation of this method demonstrated its suitability for large scale studies including extraction, FIA-FTMS analysis, and data evaluation although a high variation was observed for some samples.

Despite thorough mechanical homogenization, fecal samples are suspensions, and a lack of homogeneity can lead to deviations. Therefore, various experiments were performed to evaluate the underlying factors including centrifugation, bead beating, variation of sample concentration and volume, time of lipid extraction, ultrasonication, stool grade, and storage of extracted samples.
Centrifugation of samples showed that DG species were present in similar concentrations in the supernatant as well as in the pellet, whereas TG species were detected predominantly in the pellet. Additional homogenization using Precellys® homogenizer resulted in a slight decrease in CV for TG species, whereas the CV for DG species remained constant or even slightly increased. Variation of sample concentration did not reduce variations. Furthermore, no improvement in reproducibility could be achieved by changing the incubation time during extraction or by ultrasonication. Analysis of the consistency of fecal material provided no clear evidence for correlation of CVs and stool grade.
Only extraction of a higher sample volume resulted in lower CVs for both DG and TG species. After extraction samples could be stored until measurement for about one week. Storage temperature seemed to have little influence on the concentrations. However, it is recommended to store the samples at least at -20°C to avoid possible changes due to lipolysis.

In summary, these data clearly demonstrate that homogenization is very important for reproducibility and accuracy of lipid quantification of human fecal material. Despite extensive testing, variation of concentrations for some samples was substantial. Of note, lipid species profiles seem to be largely unaffected by homogenization and extraction conditions.

3.1.5 Pre-Analytics

Suitable pre-analytics is an important prerequisite for meaningful medical laboratory diagnostic. Many factors before, during and after sampling can have an influence on the test results. Storage of the samples and their transport to the laboratory are also important factors influencing the laboratory analysis. In practice, it is often the case that the raw fecal material collected from patients is not directly subjected to analysis but stored and transported for substantial times. In many cases the material is not sufficiently cooled (2 - 8°C). Hence, it is important to find a suitable additive as a stabiliser to prevent changing of analyte concentrations, e.g. by enzymatic reactions.

3.1.5.1 Effect of Solvents

In order to ensure the stability of the fecal material, we checked whether the solvent used for sample preparation may affect DG/TG concentrations. In several studies homogenization of fecal material was performed in water (172) or aqueous buffer (173, 174) but also in diluted organic solvents (175, 176). In our laboratory, diluted isopropanol was used to stabilize fecal concentrations of short chain fatty acids (177), thus the effect of isopropanol was investigated for DG/TG concentrations. Therefore, fecal raw material was homogenized in water and subsequently diluted 3- to 7-fold (by volume) with either water or isopropanol (Figure 3.8) and immediately stored at -80°C. Unexpectedly, addition of isopropanol tremendously increased DG concentrations in almost all samples. Moreover, in two of the six samples, we observed a drop of TG concentrations. However, the increase of DG could not be explained by TG degradation in these samples because the increase of DG exceeded the decreased amount of TG.

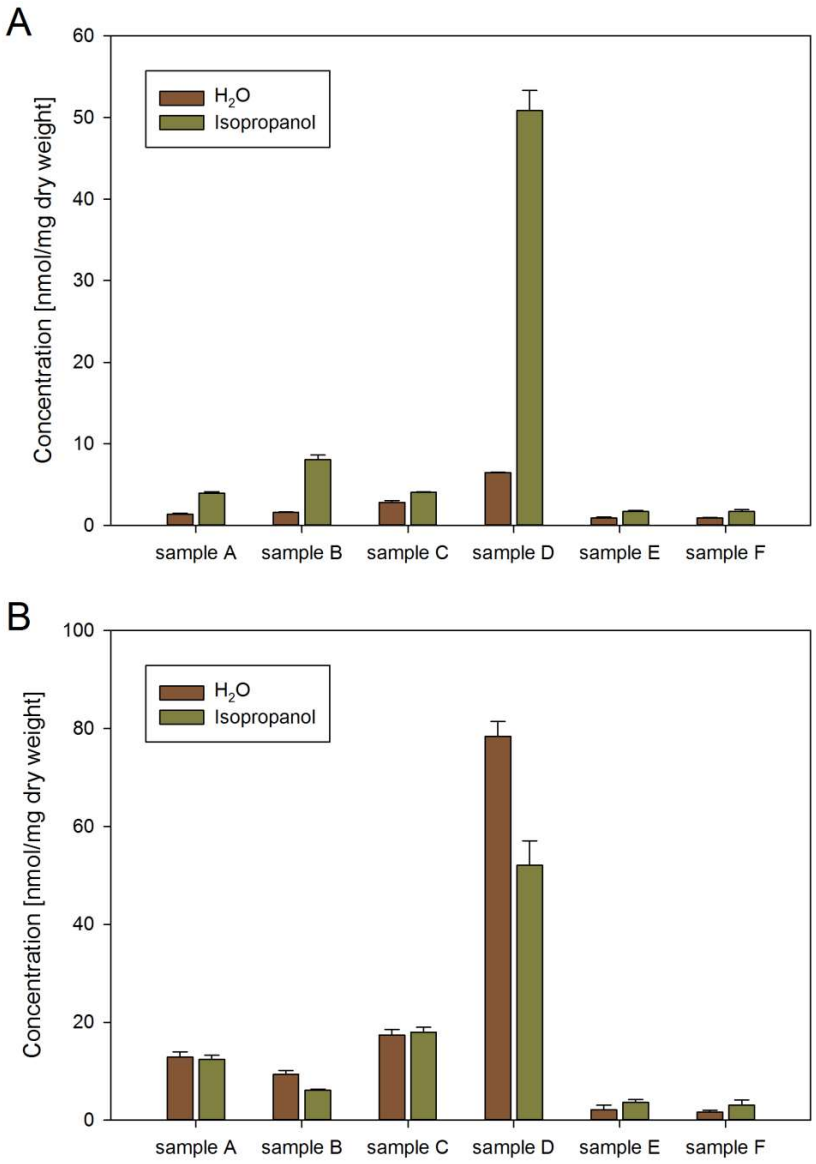


Figure 3.8: Effect of isopropanol addition on DG (A) and TG (B) quantification. Six individual samples homogenized in water and supplemented with the same volume of either H_2O (brown) or isopropanol (green) (70% related to volume) are displayed.

Comparison of spectra of samples stabilized in water or isopropanol showed clear differences in all DG species profiles and in the TG profiles of three of the six samples (Table 3.21 and Table 3.22). We could not observe additional species upon isopropanol addition and no common pattern in the increased DG species for the individual samples.

Table 3.21: Species profile in % total DG for aqueous and isopropanol-containing samples (Figure 3.8).

Species	sample A		sample B		sample C		sample D		sample E		sample F	
	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]
DG 34:3	0.6	0.6	0.0	0.4	1.0	0.9	1.3	0.9	2.1	1.6	8.7	5.3
DG 34:2	7.1	5.9	19.2	12.8	10.8	8.6	6.1	7.2	8.4	8.2	10.0	10.7
DG 34:1	8.6	7.0	9.5	5.7	13.5	11.8	4.5	1.9	8.1	7.8	19.1	15.0
DG 36:5	1.3	1.4	1.1	1.7	2.5	2.3	3.7	2.1	3.9	7.9	9.1	4.5
DG 36:4	13.3	13.0	23.5	27.1	18.3	16.3	24.4	43.6	26.5	20.9	16.2	22.9
DG 36:3	26.3	24.6	21.8	25.6	16.3	15.5	29.4	33.0	26.4	20.7	14.4	18.5
DG 36:2	42.8	47.5	24.9	26.7	37.5	44.6	30.6	11.3	24.7	33.0	22.4	23.1

Table 3.22: Species profile in % total TG for aqueous and isopropanol-containing samples (Figure 3.8).

Species	sample A		sample B		sample C		sample D		sample E		sample F	
	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]	H_2O [%]	Isopropanol [%]
TG 52:4	3.5	3.8	14.1	14.4	5.0	4.9	5.2	8.4	6.2	9.3	4.5	7.5
TG 52:3	4.8	5.1	8.7	8.4	3.8	3.8	5.0	5.1	3.4	6.3	6.0	5.9
TG 52:2	9.8	10.0	7.2	7.2	12.1	12.2	4.3	2.1	10.2	6.9	15.4	11.9
TG 54:7	0.6	0.8	1.5	1.6	1.4	1.6	4.2	5.7	9.0	7.6	1.5	1.5
TG 54:6	5.0	5.1	17.1	16.7	7.1	7.4	11.8	22.0	7.5	14.0	8.3	13.8
TG 54:5	9.9	9.3	16.4	15.8	5.4	5.6	23.3	32.5	8.3	14.5	7.3	11.0
TG 54:4	23.0	22.5	14.0	13.6	13.0	12.9	23.4	18.0	11.8	14.6	14.2	14.0
TG 54:3	43.5	43.4	20.9	22.4	52.1	51.6	22.7	6.3	43.6	26.8	42.9	34.3

To get more insight, we examined both aqueous and isopropanol containing sample homogenates by light microscopy (Figure 3.9). Clearly, aqueous samples seemed to be more homogeneous compared to isopropanol containing samples. However, in aqueous samples a massive presence of bacteria could be observed. To inhibit metabolic activity and to reduce health risks, fecal samples are frequently treated with alcohols.

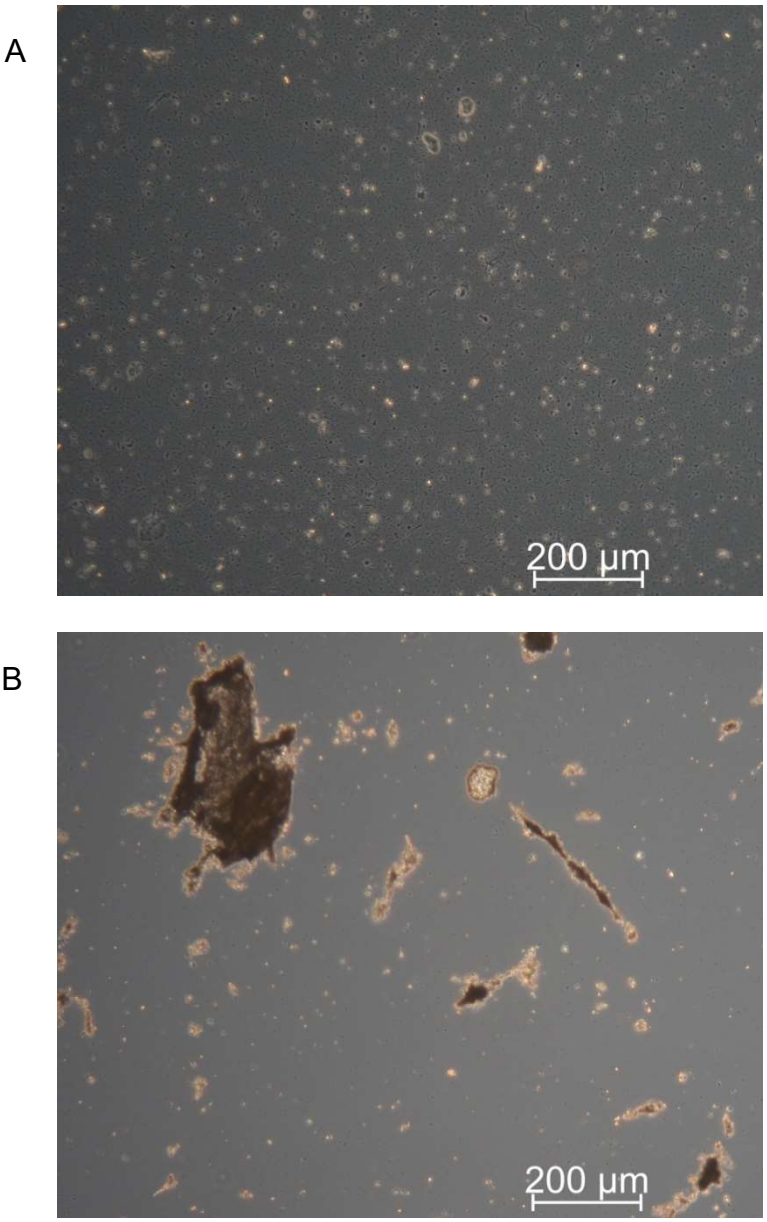


Figure 3.9: Comparison of human fecal sample D diluted either in water (A) or isopropanol (B) at a dry weight of 2.0 mg dw/mL using phase contrast microscopy with 10 x magnification.

In a next step, we checked whether there is a trend regarding the alcohols used (methanol < ethanol < isopropanol) for stabilization. Therefore, the raw fecal material of sample C was thawed and a part of it was homogenized and additionally dissolved in either MeOH or EtOH. Results are shown in Table 3.23.

Table 3.23: Species profile in % total of DG and TG for sample C either in H_2O, methanol, ethanol, or isopropanol. Samples were measured in triplicates.

	Species	H_2O Mean (n=3) [%]	methanol Mean (n=3) [%]	ethanol Mean (n=3) [%]	isopropanol Mean (n=3) [%]
Diacylglyerol	DG 34:3	0.9	0.6	0.6	0.6
	DG 34:2	8.9	9.0	8.8	8.6
	DG 34:1	4.1	3.5	3.7	3.6
	DG 36:5	0.4	0.4	0.3	0.4
	DG 36:4	38.0	42.2	42.1	42.6
	DG 36:3	34.7	32.4	32.8	32.6
	DG 36:2	13.0	11.9	11.7	11.6
Triacylglycerol	TG 50:2	0.8	0.8	0.8	0.8
	TG 50:1	0.2	0.2	0.2	0.2
	TG 50:0	0.2	0.1	0.1	0.2
	TG 52:5	0.4	0.3	0.3	0.4
	TG 52:4	8.8	8.9	8.9	8.9
	TG 52:3	5.5	5.4	5.4	5.5
	TG 52:2	1.9	1.8	1.8	1.9
	TG 54:7	0.4	0.4	0.4	0.4
	TG 54:6	26.1	26.4	26.7	26.6
	TG 54:5	34.2	34.3	34.3	33.9
	TG 54:4	16.1	16.0	15.7	16.0
	TG 54:3	5.6	5.4	5.3	5.4

For this sample, the data did not show any trend regarding the series of alcohol. Here, species profiles showed only small changes between the solvents, for both DG and TG species. This could be already demonstrated for sample C in Figure 3.8. DG species with 36 carbon atoms differed slightly in H_2O compared to the alcohols used. This trend could not be observed for TG species with 54 carbon atoms. In case of species profiles, the choice of solvent seemed to be independent of the detected concentration.

For further solvent comparison, a fresh native sample was homogenized in H_2O as described above and dried under vacuum overnight. Samples were dissolved in either 100 µL H_2O, MeOH, EtOH, or isopropanol and a standard mix was spiked to the different solvents' prior extraction. The results are shown in Table 3.24. Concentration of DG and TG could be detected at similar levels for all solvents. For both lipid classes, CV showed less than 10% for all solvents, being highest for H_2O. The different concentrations of lipids contained in samples from previous experiments could not be explained and therefore may already occur during homogenization of the raw fecal material.

Table 3.24: Calculated CV of DG and TG sum concentrations of a sample dried for extraction overnight prior addition of different solvents. Reconstituted samples were measured in triplicates.

	Diacylglycerol		Triacylglycerol	
solvents	Mean (n=3) [nmol/mg dw]	CV [%]	Mean (n=3) [nmol/mg dw]	CV [%]
H_2O	**1.85**	9.2	**6.00**	10.8
methanol	**2.11**	4.1	**6.63**	7.2
ethanol	**2.06**	3.4	**6.71**	7.8
isopropanol	**2.01**	3.5	**6.01**	5.1

3.1.5.2 Effect of Storage Temperature

Next, we asked whether there is a suitable solvent for stabilizing the fecal material before and after homogenization. For this experiment the same samples were used as described for the previous tests. The raw fecal material was collected in the morning and immediately brought to the laboratory including sufficient cooling, where it was instantly homogenized. Homogenization was carried out using the pre-analytics protocol described under *2.5.1 Feces Homogenization*. Homogenized samples were then dissolved in various solvents: H_2O, methanol, ethanol, or isopropanol. Some samples were immediately stored at -80°C while the remaining samples were stored at either 4°C or at RT for 30 minutes to 4 days until freezing at -80°C.

In a first preliminary test feces of two different donors were dissolved in H_2O, methanol, or isopropanol, respectively. DG species have been found to be relatively stable up to four days, with only minor variation that did not follow a clear trend. Concentration of TG species decreased rapidly after 3 h. The CVs were mostly below 15%. Some outliers were observed. Species profiles in %, shown in Table 3.25 - Table 3.28, represent the most common DG and TG species in fecal material for each sample. The differences between the respective times and temperatures were much less, the percentages fluctuated only slightly in some cases. However, there were several exceptions, especially when samples were stored at 4°C or RT for more than one day. Methanol seemed to be less stable than H_2O or isopropanol.
From this it could be concluded that the total concentrations of the two lipid classes changed over time, whereas the proportions of the respective species only slightly changed.

Table 3.25: Species profile in % total DG. Data of sample A for either H_2O, methanol or isopropanol are displayed.

Sample A													
H_2O													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	0.3	0.3	0.1	0.4	0.2	0.6	8.9	0.4	0.4	0.3	0.5	0.7	0.3
DG 34:2	10.5	10.1	10.5	10.2	10.5	12.1	15.4	10.8	9.4	10.5	10.7	10.7	10.5
DG 34:1	3.9	4.3	4.2	3.4	3.3	2.9	31.2	2.8	2.5	2.7	2.4	2.7	2.2
DG 36:5	0.5	0.8	0.7	0.5	0.4	1.5	n.d.	0.9	1.1	1.1	1.2	1.4	1.1
DG 36:4	46.7	44.7	45.2	47.3	46.7	47.2	10.2	47.0	46.6	47.5	47.5	47.3	49.3
DG 36:3	28.1	29.4	28.8	28.9	29.0	26.7	16.0	28.4	30.0	27.7	27.9	27.3	26.8
DG 36:2	9.9	10.4	10.4	9.4	10.0	8.9	18.3	9.6	10.0	10.3	9.9	9.9	9.8

Sample A													
Methanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	0.8	0.9	1.8	2.2	2.0	2.3	8.7	1.3	1.8	1.3	2.0	2.6	6.1
DG 34:2	11.4	11.2	11.0	10.8	11.0	12.0	5.0	10.8	10.5	10.1	10.6	10.2	11.5
DG 34:1	2.0	2.1	1.6	1.6	1.4	1.5	19.4	0.7	1.5	1.8	1.9	3.0	1.8
DG 36:5	1.6	1.5	3.9	4.8	5.7	7.3	6.1	4.6	4.2	4.1	4.1	4.7	6.9
DG 36:4	45.3	44.7	45.8	40.2	35.9	21.1	53.1	45.1	44.3	43.1	37.7	27.0	13.7
DG 36:3	29.4	30.0	30.1	34.3	36.0	45.9	2.4	31.9	31.8	32.0	34.0	41.7	48.5
DG 36:2	9.5	9.8	5.9	6.1	8.1	9.9	5.4	5.7	6.0	7.7	9.7	10.8	11.6

Sample A													
Isopropanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	0.6	0.6	0.6	0.6	0.7	0.7	1.6	0.6	0.6	0.6	0.6	0.7	0.7
DG 34:2	10.3	10.1	10.1	10.5	10.5	10.9	19.8	10.6	10.3	10.6	10.2	10.5	10.4
DG 34:1	1.6	1.4	1.5	1.5	1.4	1.3	38.4	1.6	1.5	1.6	1.6	1.4	1.4
DG 36:5	1.1	1.1	1.2	1.2	1.3	1.3	1.1	1.2	1.1	1.1	1.2	1.3	1.3
DG 36:4	49.5	50.7	50.2	50.2	49.9	50.6	11.1	49.7	50.4	50.1	50.4	50.8	51.2
DG 36:3	28.4	28.4	28.3	28.4	28.1	26.8	14.6	27.8	28.1	27.4	27.7	27.1	27.2
DG 36:2	8.5	7.6	8.1	7.5	8.2	8.3	13.4	8.5	7.9	8.5	8.3	8.2	7.8

Table 3.26: Species profile in % total DG. Data of sample B for either H_2O, methanol or isopropanol are displayed.

Sample B													
H_2O													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	0.8	0.8	0.8	0.6	0.7	0.5	0.6	0.8	0.8	1.0	1.1	1.0	1.0
DG 34:2	7.0	5.7	6.2	7.9	7.9	7.2	8.4	5.9	5.1	7.6	7.9	9.2	9.0
DG 34:1	2.8	2.5	2.5	2.7	2.7	2.7	2.7	2.4	2.4	2.7	2.8	3.1	3.1
DG 36:5	4.7	6.4	5.3	3.5	3.9	3.9	3.1	7.0	8.2	4.9	5.2	3.9	3.6
DG 36:4	37.2	36.9	37.7	37.5	39.3	37.6	38.7	37.5	36.6	38.2	38.7	39.4	40.2
DG 36:3	32.6	33.6	33.0	32.1	30.3	32.0	31.1	33.1	33.6	30.9	30.0	27.9	28.2
DG 36:2	14.8	14.1	14.5	15.6	15.2	16.1	15.4	13.2	13.4	14.6	14.3	15.5	15.0

Sample B													
Methanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	2.4	2.5	2.3	2.4	2.3	2.1	1.5	2.5	2.4	2.2	2.0	1.6	2.0
DG 34:2	9.1	8.9	8.5	8.7	8.6	10.3	13.3	8.6	8.8	8.9	8.6	8.8	9.9
DG 34:1	1.9	2.0	2.0	2.0	2.5	5.6	11.4	1.6	1.8	2.0	1.7	2.1	3.5
DG 36:5	7.2	7.4	7.6	7.8	7.4	5.2	1.2	7.9	7.2	6.7	7.3	6.2	4.6
DG 36:4	49.7	51.0	51.1	50.2	48.6	40.2	39.9	51.6	51.6	50.0	53.4	52.9	43.5
DG 36:3	22.1	21.1	21.5	21.8	22.6	25.0	24.3	21.2	21.4	22.2	20.8	21.6	26.4
DG 36:2	7.6	7.2	6.9	7.0	7.9	11.6	8.5	6.5	6.9	7.9	6.2	6.7	10.1

Sample B													
Isopropanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
DG 34:3	1.2	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.2	1.2	1.2	1.2	1.2
DG 34:2	8.6	8.6	8.5	8.4	8.4	8.6	9.1	8.6	8.6	8.5	8.4	8.3	8.3
DG 34:1	2.3	2.3	2.2	2.3	2.2	2.3	2.6	2.2	2.2	2.2	2.3	2.1	2.1
DG 36:5	3.4	3.3	3.2	3.3	3.4	3.3	3.6	3.3	3.2	3.3	3.1	3.3	3.3
DG 36:4	44.8	44.8	44.8	44.8	44.7	43.5	42.3	45.1	44.9	45.2	44.3	45.1	45.3
DG 36:3	26.5	26.5	26.8	26.7	27.3	27.9	27.8	26.4	26.7	26.5	26.9	26.7	26.9
DG 36:2	13.2	13.4	13.2	13.4	13.0	13.2	13.4	13.3	13.1	13.2	13.8	13.3	12.9

Table 3.27: Species profile in % total TG. Data of sample A for either H_2O, methanol or isopropanol are displayed.

	Sample A												
	H_2O												
	-80°C	**4°C**						**RT**					
Species		30 minutes	60 minutes	180 minutes	360 minutes	1 day	3 days	30 minutes	60 minutes	180 minutes	360 minutes	1 day	3 days
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
TG 50:2	1.4	1.3	1.3	1.2	1.1	2.9	4.4	1.2	1.1	1.2	1.1	1.2	1.2
TG 50:1	0.9	0.5	0.6	0.5	0.5	0.1	6.4	0.4	0.3	0.4	0.3	0.2	0.2
TG 52:5	0.5	0.5	0.5	0.5	0.5	1.3	71.4	0.5	0.5	0.5	0.6	0.6	0.5
TG 52:4	27.5	21.8	26.0	24.9	22.5	19.7	n.d.	18.9	15.1	16.6	16.3	13.6	16.0
TG 52:3	2.3	3.2	2.5	2.6	2.9	3.8	2.4	3.7	4.5	4.3	4.1	4.8	4.6
TG 52:2	2.3	2.0	2.2	1.9	1.8	1.4	4.0	1.6	1.5	1.5	1.5	1.6	1.5
TG 54:7	0.6	1.0	0.7	0.5	0.6	1.7	2.0	0.7	0.7	0.9	0.8	0.9	0.9
TG 54:6	26.5	28.3	26.8	28.2	29.3	31.0	2.6	30.7	30.4	31.4	31.6	30.7	31.1
TG 54:5	24.8	26.6	25.4	26.6	27.3	25.5	3.3	28.5	30.8	29.6	29.8	29.7	29.1
TG 54:4	10.2	11.2	10.5	10.3	10.6	10.1	1.7	11.1	12.3	11.2	11.2	12.6	11.7
TG 54:3	2.9	3.7	3.6	2.7	2.9	2.4	1.8	2.8	2.9	2.6	2.7	4.0	3.2

Sample A													
Methanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
TG 50:2	1.6	1.4	2.1	2.8	2.7	3.6	5.9	2.0	1.8	1.6	1.7	2.1	3.0
TG 50:1	0.2	0.1	1.1	1.9	2.0	3.4	7.8	0.8	0.8	0.6	0.5	0.7	3.5
TG 52:5	0.8	0.7	0.8	0.7	0.7	0.5	n.d.	0.6	0.6	0.5	0.5	0.7	0.2
TG 52:4	12.8	12.1	19.1	26.7	29.6	50.5	30.2	19.0	16.8	13.3	13.5	14.2	53.1
TG 52:3	5.9	5.9	5.4	4.1	3.7	0.2	2.9	4.9	5.5	6.0	6.4	7.4	n.d.
TG 52:2	2.3	2.0	3.3	4.7	5.1	6.6	33.1	3.1	3.0	2.7	3.0	3.2	5.7
TG 54:7	1.2	1.7	1.2	1.0	1.0	0.1	0.9	0.8	0.8	0.7	0.8	1.2	1.5
TG 54:6	28.9	28.6	24.8	19.7	16.8	9.9	3.7	25.7	25.7	25.5	23.2	21.6	7.0
TG 54:5	29.3	30.9	25.5	22.3	20.5	12.1	4.7	26.3	27.6	30.0	29.5	27.6	11.1
TG 54:4	12.8	12.8	12.2	11.1	12.1	8.0	5.8	12.5	13.3	14.5	15.6	15.7	8.9
TG 54:3	4.3	3.7	4.4	5.0	5.9	5.3	5.0	4.2	4.2	4.7	5.1	5.6	6.0

Sample A													
Isopropanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
TG 50:2	1.4	1.3	1.4	1.4	1.3	1.6	0.0	1.4	1.3	1.4	1.4	1.3	1.6
TG 50:1	0.1	0.1	0.1	0.1	0.1	0.4	25.6	n.d.	n.d.	n.d.	0.1	0.1	0.6
TG 52:5	0.8	0.9	0.9	0.9	0.9	1.1	n.d.	0.9	0.9	1.0	1.1	1.2	1.3
TG 52:4	13.1	13.2	13.3	13.4	13.3	13.6	n.d.	13.4	13.3	13.7	13.6	13.8	13.7
TG 52:3	4.9	4.4	4.6	5.2	4.4	5.1	8.4	4.6	4.4	4.4	4.7	4.2	4.7
TG 52:2	1.3	1.2	1.3	1.3	1.3	1.6	12.2	1.1	1.1	1.2	1.1	1.1	1.5
TG 54:7	1.4	1.5	1.5	1.4	1.6	1.8	4.5	1.6	1.7	1.7	1.7	2.2	2.1
TG 54:6	34.0	36.3	34.5	33.2	35.1	34.7	15.5	34.9	35.8	36.0	35.2	37.8	36.1
TG 54:5	30.4	29.6	29.9	29.8	29.4	27.4	19.1	30.0	30.0	28.9	29.6	26.2	24.6
TG 54:4	9.8	9.0	10.0	10.7	9.9	9.1	7.3	9.6	9.0	9.1	9.3	8.8	8.8
TG 54:3	2.8	2.6	2.7	2.6	2.7	3.5	7.4	2.5	2.5	2.5	2.3	3.3	5.1

Table 3.28: Species profile in % total TG. Data of sample B for either H_2O, methanol or isopropanol are displayed.

	Sample B												
	H_2O												
	-80°C				**4°C**						**RT**		
Species		30 minutes	60 minutes	180 minutes	360 minutes	1 day	3 days	30 minutes	60 minutes	180 minutes	360 minutes	1 day	3 days
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
TG 50:2	0.9	0.9	0.9	0.9	0.9	0.9	1.0	1.0	0.8	0.9	1.0	1.3	1.0
TG 50:1	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.1	0.2
TG 52:5	0.5	0.7	0.6	0.4	0.4	0.4	0.5	0.6	0.9	0.6	0.5	0.8	0.5
TG 52:4	9.2	9.2	9.3	10.1	10.1	8.5	10.2	10.5	8.5	9.2	10.0	11.1	10.2
TG 52:3	5.7	5.5	5.6	5.5	5.0	5.0	5.7	5.1	5.6	5.5	5.8	5.2	5.5
TG 52:2	2.9	2.8	2.6	2.6	2.4	3.8	2.9	2.2	2.4	3.5	2.6	3.0	2.7
TG 54:7	1.3	3.4	2.0	0.7	0.7	1.0	0.9	1.5	3.9	1.6	1.5	2.2	1.1
TG 54:6	25.1	24.1	24.5	26.0	28.4	22.9	24.9	29.3	23.9	24.0	24.8	24.7	26.6
TG 54:5	28.9	28.1	28.9	29.3	29.3	24.9	28.0	29.0	29.5	26.9	29.6	26.9	28.4
TG 54:4	16.0	16.0	16.6	15.2	15.4	13.8	15.6	13.9	16.8	15.3	15.4	14.6	15.2
TG 54:3	9.4	9.0	8.7	9.1	7.2	18.7	10.1	7.0	7.4	12.1	8.6	10.0	8.7

Sample B													
Methanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
TG 50:2	5.5	9.5	2.9	10.4	5.3	1.8	6.8	14.2	1.6	1.7	9.0	5.5	1.2
TG 50:1	0.5	0.6	0.4	0.6	0.8	0.4	1.0	0.8	0.2	0.2	1.4	0.9	0.3
TG 52:5	1.4	1.9	0.8	1.4	1.3	0.9	0.3	2.3	1.2	1.0	1.7	0.9	0.5
TG 52:4	12.5	15.8	9.3	14.1	14.1	10.4	30.9	17.7	10.4	10.1	14.8	13.5	10.3
TG 52:3	6.5	6.8	6.2	6.3	6.3	5.6	2.5	7.0	6.3	6.5	6.4	6.8	6.8
TG 52:2	2.9	3.3	4.0	3.3	3.9	4.6	3.7	3.8	2.6	2.6	4.1	3.5	3.1
TG 54:7	3.5	n.d.	1.7	11.2	n.d.	0.9	14.5	n.d.	5.5	2.5	n.d.	n.d.	0.9
TG 54:6	25.1	24.3	21.5	17.5	22.6	22.9	10.1	19.8	23.4	24.3	20.3	19.6	22.4
TG 54:5	22.9	19.5	26.2	17.2	21.7	21.5	14.3	16.1	25.7	28.3	19.6	22.8	28.4
TG 54:4	11.8	10.8	14.9	9.1	12.6	13.0	9.2	10.2	13.9	15.2	12.6	14.7	16.8
TG 54:3	7.5	7.4	12.1	8.9	11.5	18.1	6.6	8.2	9.1	7.6	9.9	11.8	9.3

Sample B													
Isopropanol													
	-80°C				**4°C**						**RT**		
Species	[%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]	30 minutes [%]	60 minutes [%]	180 minutes [%]	360 minutes [%]	1 day [%]	3 days [%]
TG 50:2	1.1	1.0	1.0	1.0	1.1	1.1	3.0	1.0	1.0	1.1	1.0	1.0	1.0
TG 50:1	0.2	0.2	0.1	0.2	0.2	0.4	5.7	0.1	0.1	0.1	0.1	0.2	0.4
TG 52:5	0.8	0.8	0.8	0.9	0.9	1.0	4.4	0.9	0.9	0.9	0.9	1.1	1.3
TG 52:4	10.9	10.6	10.7	10.8	11.3	10.9	10.3	10.8	10.8	10.8	10.7	11.2	11.3
TG 52:3	5.2	5.1	5.1	5.1	5.3	5.2	5.8	5.1	4.9	4.7	4.5	4.0	4.1
TG 52:2	2.5	2.5	2.4	2.4	2.4	2.4	3.4	2.4	2.3	2.3	2.1	1.9	1.5
TG 54:7	3.4	3.4	3.4	3.6	3.9	4.3	7.3	3.5	3.5	3.7	4.1	5.1	5.8
TG 54:6	26.8	26.5	27.1	27.9	28.4	28.4	20.3	27.6	27.5	27.9	29.2	31.9	34.3
TG 54:5	28.0	28.1	27.7	27.0	26.8	26.1	20.7	27.7	28.1	28.2	28.1	26.8	25.0
TG 54:4	13.3	13.6	13.5	13.0	12.3	12.1	11.1	13.2	13.1	12.7	12.2	10.7	10.0
TG 54:3	7.9	8.2	8.1	8.0	7.5	7.9	7.9	7.7	7.7	7.6	7.1	6.2	5.4

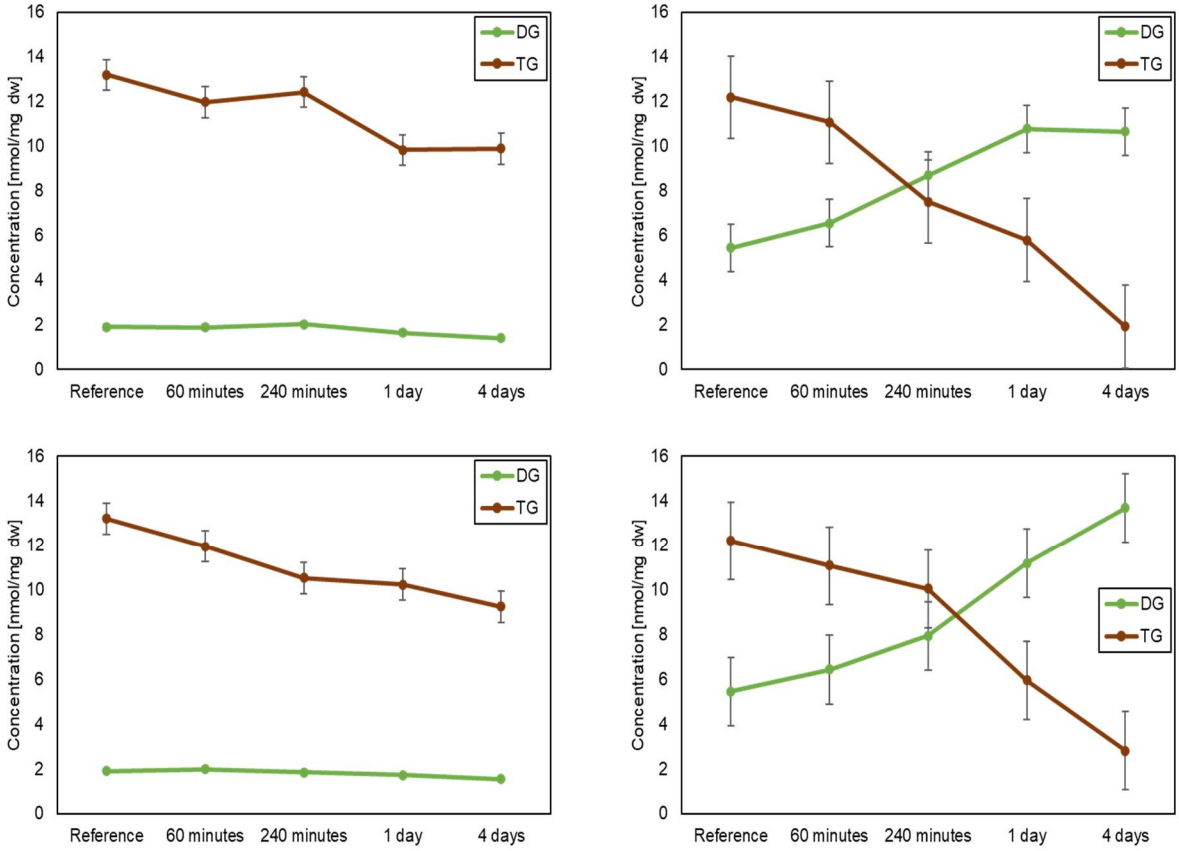


Figure 3.10: Schematic illustration of DG and TG concentrations in H_2O (left) and isopropanol (right) at either 4°C (above) or at room temperature (RT, below) between 60 minutes and 4 days. Mean values of three different samples are shown.

As the data in Table 3.21, Table 3.22 and Figure 3.8 already showed, the addition of isopropanol significantly increased DG concentrations in almost all samples. Whereas DG species seemed to be stable in water, the concentrations in isopropanol changed tremendously; in four of six samples the concentration even increased during the day. Each sample behaved differently. The storage temperature seemed to have a minor influence on the concentration, although no clear trend was apparent.

Concentration between the two solvents differed for TG species. All samples in isopropanol showed a considerable drop of TG concentration after 24 hours after homogenization. However, the concentration in water seemed to be stable for most samples (Data shown in Table 3.29, Table 3.30, and Figure 3.10). Again, the increase of DG could not be explained by TG degradation in these samples because the increase of DG exceeded the decreased amount of TG.

Table 3.29: DG concentrations of six different samples in either H_2O or isopropanol are displayed. The samples were stored at three different temperatures in a period from 60 minutes to 4 days, and immediately stored at -80°C.

		Diacylglycerol [nmol/mg dw]											
		sample A		sample B		sample C		sample D		sample E		sample F	
temperature	time	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol
-80°C		1.37	3.99	1.60	8.08	2.84	4.06	6.49	50.83	0.23	28.86	1.84	352.75
4°C	60 minutes	1.18	5.79	1.55	8.65	2.92	5.22	6.42	61.15	0.16	35.09	5.83	594.29
	240 minutes	1.62	10.49	1.51	8.35	2.98	7.21	6.46	67.23	0.18	32.41	1.49	644.35
	1 day	1.02	9.31	1.25	8.76	2.69	14.22	5.85	38.82	0.29	27.68	2.16	337.44
	4 days	0.91	8.97	1.05	7.63	2.23	15.33	5.04	28.34	0.22	2.34	1.19	108.01
RT	60 minutes	1.50	5.46	1.42	8.93	3.05	4.87	5.69	62.40	0.30	36.13	11.30	686.68
	240 minutes	1.27	8.49	1.39	8.62	2.89	6.65	5.11	67.09	0.17	34.08	4.54	536.52
	1 day	1.12	11.79	1.16	8.67	2.86	13.05	5.10	71.33	0.40	40.32	4.34	653.77
	4 days	1.13	13.68	1.10	8.78	2.38	18.53	5.34	50.90	0.16	38.37	4.15	676.08

Table 3.30: TG concentrations of six different samples in either H_2O or isopropanol are displayed. The samples were stored at three different temperatures in a period from 60 minutes to 4 days, and immediately stored at -80°C.

		Triacylglycerol [nmol/mg dw]											
		sample A		sample B		sample C		sample D		sample E		sample F	
temperature	time	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol	H_2O	Isopropanol
-80°C		12.84	12.39	9.40	6.13	17.37	17.99	78.34	52.01	0.17	8.48	5.06	70.35
4°C	60 minutes	11.11	10.22	8.79	5.07	15.99	17.91	80.78	21.35	0.19	5.56	13.35	43.65
	240 minutes	12.10	2.81	8.12	4.60	17.01	15.12	77.57	13.15	0.20	3.69	7.15	23.37
	1 day	9.00	7.78	5.65	3.35	14.81	6.25	80.14	8.24	0.38	0.44	7.33	2.74
	4 days	10.23	1.79	5.14	1.64	14.27	2.32	73.86	6.85	1.63	1.51	5.38	0.36
RT	60 minutes	12.85	11.13	6.58	5.73	16.37	16.31	71.57	27.34	0.41	7.61	10.65	68.08
	240 minutes	10.20	7.90	5.62	4.43	15.68	17.72	77.79	21.28	0.38	6.76	9.83	73.32
	1 day	10.79	4.28	3.92	4.18	15.90	9.35	71.03	22.18	0.72	1.79	15.74	24.94
	4 days	9.79	2.77	3.04	1.76	14.81	3.90	71.22	9.25	0.45	0.36	15.36	5.00

3.1.5.3 Effect of Freeze/Thaw Cycle

To investigate the reason for the increase of DG concentrations in isopropanol, a new specimen was freshly homogenized and stored again at different conditions. This time the samples were extracted immediately afterwards and the remaining sample material was stored at -80°C (except the reference samples, Table 3.31). A significant difference in concentration could already be observed in the fresh sample material and changed tremendously after freezing. Whereas the samples in H_2O, for both DG and TG species, showed an increased concentration after freezing, TG concentrations again decreased in isopropanol. Again, the aqueous samples seemed to be more stable before and after freezing.

The increase of DG upon H_2O and isopropanol addition seemed to be related to both disruption of bacteria resulting in improved extractability of DG and lipolysis of TG. The latter seemed to be triggered by addition of isopropanol in some samples and matched lipolytic activities observed in organic solvents (167, 168). These data clearly demonstrate that further studies are warranted to evaluate optimal pre-analytic conditions for fecal samples as well as the origin of these differences.

Table 3.31: Sum concentrations are listed for DG and TG species of sample C. The sample was homogenized and stored at different temperatures between 60 minutes and 4 days. Samples were extracted and measured directly after homogenization (fresh sample) as well as after freezing (sample after freezing).

		sample C							
		Diacylglycerol				Triacylglycerol			
temperature	time	fresh sample		sample after freezing		fresh sample		sample after freezing	
		H_2O [nmol/mg dw]	Isopropanol [nmol/mg dw]	H_2O [nmol/mg dw]	Isopropanol [nmol/mg dw]	H_2O [nmol/mg dw]	Isopropanol [nmol/mg dw]	H_2O [nmol/mg dw]	Isopropanol [nmol/mg dw]
-80°C		6.49	50.8	14.57	24.58	78.34	52.01	102.14	35.46
4°C	60 minutes	6.42	61.15	13.30	35.03	80.78	21.35	95.84	21.23
	240 minutes	6.46	67.23	12.09	35.32	77.57	13.15	90.48	19.03
	1 day	5.85	38.82	9.83	24.45	80.14	8.24	87.06	7.18
	4 days	5.04	28.34	9.74	24.69	73.86	6.85	74.71	4.68
RT	60 minutes	5.69	62.40	11.56	37.96	71.57	27.34	95.57	23.34
	240 minutes	5.11	67.09	11.21	34.74	77.79	21.28	83.50	17.63
	1 day	5.10	71.33	10.55	27.48	71.03	22.18	86.75	14.38
	4 days	5.34	50.90	10.40	26.80	71.22	9.25	78.46	12.90

3.1.5.4 Effect of Sample Preparation

Finally, we checked whether there is a possibility to stabilize DG and TG concentrations over several days by addition of SDS (0.1%, 1.0%, 2.0%, and 3.0%) as shown in Table 3.32. The samples were stored at different conditions again as described before. Concentrations for DG and TG decreased despite the addition of SDS; hence this detergent is not suitable as a stabilizer. Deviation of the double values of DG species was below 12% for all samples, whereas it was below 21% for TG species, indicating at least an acceptable technical reproducibility.

Table 3.32: An isopropanol containing sample was analyzed without SDS as well as after addition of four different SDS concentrations. The sample was measured twice. Mean DG and TG species concentrations and the coefficient of variation are displayed.

		Diacylglycerol	Triacylglycerol
SDS	time	Mean (n=2) [nmol/mg dw]	Mean (n=2) [nmol/mg dw]
without SDS	0 minutes	84.03	19.72
	60 minutes	88.37	14.40
	240 minutes	75.03	12.84
	1 day	60.33	9.54
	4 days	40.77	6.35
0.1%	0 minutes	88.90	15.53
	60 minutes	79.04	12.24
	240 minutes	87.84	13.32
	1 day	68.95	10.45
	4 days	51.23	5.01
1.0%	0 minutes	91.41	15.78
	60 minutes	92.38	12.38
	240 minutes	84.98	12.80
	1 day	45.69	6.99
	4 days	56.71	5.00
2.0%	0 minutes	64.82	10.31
	60 minutes	104.79	13.47
	240 minutes	92.00	24.95
	1 day	70.11	10.31
	4 days	61.03	4.94
3.0%	0 minutes	86.14	16.83
	60 minutes	94.41	14.00
	240 minutes	103.50	14.36
	1 day	69.86	9.82
	4 days	49.65	4.32

3.1.5.5 Effect of Intra-Individual Variance

As mentioned above, 20 different samples were measured in a first step and analyzed. A high heterogeneity of the samples could be observed. DG and TG species were strongly sample-dependent in the number and type of species detected. Therefore, we asked whether DG and TG species profile possibly changed for one individual person, whose raw fecal material was collected on different days (intra-individual variance).

Table 3.33: DG and TG sum concentrations of homogenized fecal material from three different, healthy voluntary donors are displayed. The raw feces were collected on five different days. Each sample was analyzed only once.

	Volunteer A		Volunteer B		Volunteer C	
	DG sum [nmol/mg dw]	TG sum [nmol/mg dw]	DG sum [nmol/mg dw]	TG sum [nmol/mg dw]	DG sum [nmol/mg dw]	TG sum [nmol/mg dw]
day 1	4.53	0.22	1.34	0.48	2.71	0.32
day 2	1.06	0.12	9.35	1.51	2.09	0.59
day 3	18.99	3.81	0.97	0.41	5.88	3.20
day 4	5.48	4.43	0.77	0.16	6.24	2.53
day 5	4.30	1.35	0.95	0.97	4.30	0.42

For this experiment samples were collected and analyzed from three voluntary donors on five different days. The sum concentrations for DG and TG of the respective donors are shown in Table 3.33. For volunteer A, for example, the DG concentration on day 3 was three times higher in comparison to the other days. This could also be seen for volunteer B and C. Even the species profile in % (Table 3.34 and Table 3.35) showed significant variations for both DG and TG species. Since this experiment did not provide any information regarding diet, medication, or stool grades, it is difficult to determine the exact cause of these deviations. For subsequent experiments dealing with this topic, potential influence factors should be recorded in order to draw exact conclusions about the intra-individual fecal lipidome.

Table 3.34: Species profile in % total DG of the data listed in Table 3.33.

Species	Volunteer A					Volunteer B					Volunteer C				
	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]
DG 34:3	0.6	2.0	0.1	0.2	0.6	1.3	0.5	2.0	3.2	2.3	1.8	2.3	1.9	1.6	1.4
DG 34:2	11.7	12.0	2.2	2.6	6.3	9.4	7.0	9.2	5.8	7.9	11.4	11.9	8.9	6.4	13.7
DG 34:1	2.2	22.4	9.0	9.5	7.6	7.2	4.9	8.1	12.7	8.9	5.3	4.9	3.7	6.2	2.8
DG 36:5	0.7	1.8	0.1	0.2	1.2	1.7	1.1	3.7	5.0	4.0	8.8	8.7	5.7	4.0	4.4
DG 36:4	52.0	27.6	4.3	4.9	18.7	30.9	26.9	29.2	46.2	34.7	38.5	40.7	40.2	31.1	43.9
DG 36:3	22.3	13.7	17.3	16.5	24.4	28.8	27.4	18.6	10.3	19.3	22.7	20.2	25.5	21.2	23.8
DG 36:2	10.5	20.6	66.9	66.1	41.3	20.7	32.2	29.1	16.8	22.9	11.6	11.4	14.1	29.5	10.0

Table 3.35: Species profile in % total TG of the data listed in Table 3.33.

Species	Volunteer A					Volunteer B					Volunteer C				
	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]	day 1 [%]	day 2 [%]	day 3 [%]	day 4 [%]	day 5 [%]
TG 50:2	2.3	6.7	0.4	0.5	0.8	1.2	1.2	2.7	2.2	5.3	2.5	2.3	1.4	1.2	2.6
TG 50:1	2.6	15.5	1.0	1.3	1.6	5.9	0.9	5.3	7.7	27.3	2.1	1.8	0.5	1.8	1.2
TG 50:0	6.8	29.9	0.0	0.5	1.6	56.0	0.2	35.1	51.6	31.9	2.5	2.1	n.d.	n.d.	0.8
TG 52:3	5.6	2.6	3.3	3.4	4.0	3.0	5.8	4.8	0.0	1.9	8.0	6.7	6.9	4.5	8.3
TG 52:2	3.4	14.9	11.4	11.9	11.4	6.4	8.3	8.8	7.4	5.2	5.8	4.7	4.1	14.1	4.4
TG 54:7	0.9	n.d.	0.1	0.0	0.3	0.1	0.5	0.7	0.2	0.7	8.7	10.0	4.4	2.2	4.5
TG 54:6	30.2	7.7	1.5	1.0	3.1	3.4	10.7	3.4	2.3	4.7	21.1	20.1	27.6	6.8	23.5
TG 54:5	23.5	7.7	4.9	4.4	7.7	6.8	18.1	6.2	3.4	5.7	22.5	16.4	26.3	8.3	27.1
TG 54:4	17.7	6.9	18.0	16.9	18.9	8.5	16.8	7.8	7.4	6.6	14.9	17.1	17.3	12.1	18.4
TG 54:3	7.0	8.1	59.4	60.1	50.6	8.7	37.5	25.2	17.8	10.7	11.9	18.8	11.5	49.0	9.2

3.1.5.6 Evaluation of Background

Finally, the influence of different tubes used during homogenization (gentleMACS™ tubes, Sarstedt tubes, and Eppendorf tubes) was investigated for different solvents. None of the materials contained larger amounts of DG and TG species and therefore could be further used for homogenization (data not shown). Internal standard blanks were used for background correction. The solvents used for analysis (H_2O, MeOH, EtOH, and isopropanol) were spiked with a defined amount of IS and subjected to the described extraction without matrix. None of the solvents used showed significant concentrations for lipid species found in fecal material (data not shown). Therefore, an influence of the background for all solvents could be excluded.

3.1.5.7 Summary – Pre-Analytics

Currently, we cannot explain the aggregation induced by addition of isopropanol, as mentioned before. The increase of DG upon isopropanol addition seemed to be related to both lipolysis of TG species and disruption of bacteria resulting in improved extractability of DG species.

Concentrations of DG and TG species in fecal material were stable in H_2O for at least one day at RT, whereas in isopropanol the concentration already changed after one to three hours.

Besides that, preliminary data showed that already one freeze-thaw cycle seemed to influence the concentrations of both lipid classes. Homogenization in H_2O showed higher concentrations after freezing, possibly due to increased extractability.

Using SDS as stabilizer did not stabilize concentrations substantially.

Together, these data suggest that for DG/TG quantification fecal samples should be collected natively and homogenized in water but not in isopropanol. Clearly, further experiments are needed to evaluate pre-analytics in more detail.

Preliminary data suggest a high intra-individual variance of both DG and TG concentrations and species profiles. This may be related to diet but clearly needs further evaluation.

In summary, these data clearly demonstrate that pre-analytics is very important for stability, homogenization, and analysis of human fecal material and that further studies are warranted to changes in DG/TG concentrations.

3.2. Lipid Species Profile of Human Fecal Samples in Negative Ion Mode

In order to obtain better results for *lyso*-phospholipids and glycerophospholipids, further tests were carried out to enhance ionisation in negative ion mode. Extraction of the samples was again performed according to the protocol by Bligh and Dyer. Samples were resuspended using different solvents and additives before measurement on the Q Exactive Orbitrap. Table 3.36 lists the solvent combinations used with the corresponding additives and additive concentrations. In a first step, an internal standard mix (see Table 3.37) was analyzed using different solvents: LM1 (178), LM3 a/b (179), LM4 (180), and LM6 (124). Instead of ammonium acetate as described in (178), LM1 was spiked with ammonium formate.

Table 3.36: Comparison of different solvents, additives, and concentrations for measurements in negative ion mode. Solvents containing methylamine were either prepared from aqueous (a) or EtOH-containing (b) solutions. Experiments using LM2 (MeOH/$CHCl_3$ (5:1 v/v)) were performed in different concentration of methylamine solution.

	Solvent	**Additive**	**Concentration**
LM1	2-propanol/MeOH/H_2O (8:5:1 v/v/v)	ammonium formate	7.5 mM
LM2 a/b	2-propanol/MeOH/H_2O (8:5:1 v/v/v)	methylamine solution a) H_2O b) EtOH	0.005 %
LM3 a/b	MeOH/$CHCl_3$ (5:1 v/v)	methylamine solution a) H_2O b) EtOH	0.005 % 0.01 % 0.05 % 0.1 %
LM4	EtOH/$CHCl_3$ (4:1 v/v)	trimethylamine solution EtOH	0.1 %
LM5 a/b	EtOH/$CHCl_3$ (4:1 v/v)	methylamine solution a) H_2O b) EtOH	0.005 %
LM6	2-propanol/MeOH/$CHCl_3$ (4:2:1 v/v/v)	ammonium formate	7.5 mM
LM7 a/b	2-propanol/MeOH/$CHCl_3$ (4:2:1 v/v/v)	methylamine solution a) H_2O b) EtOH	0.005 %

Table 3.37: Internal Standard (IS) including respective *m/z* values for $[M-H]^-$ ions applied in negative ion mode analysis, as well as the intensities obtained from different solvents (LM1, LM3 a, LM3 b, LM4, and LM6) are displayed. Methylamine concentration was set to 0.005% for LM3 a and LM3 b.

Species	*m/z* $[M-H]^-$	LM1	LM3 a	LM3 b	LM4	LM6
FA 13:0	213.1860	1.64E+05	1.20E+06	2.94E+07	3.73E+05	5.17E+04
FA 18:0	283.2643	4.96E+06	1.34E+08	1.75E+08	1.98E+07	1.01E+07
FA 18:2	279.2330	1.82E+07	2.41E+08	3.18E+08	3.19E+07	2.41E+07
FA 20:4	303.2330	8.50E+07	2.65E+08	3.48E+08	4.23E+07	8.43E+07
FA 23:0	353.3425	7.14E+05	2.49E+06	6.61E+07	8.30E+05	2.51E+04
MG 13:0	287.2227	6.26E+05	7.97E+06	1.94E+06	6.46E+05	4.67E+04
MG 19:0	371.3167	5.19E+06	2.57E+07	5.87E+06	1.51E+06	1.25E+06
DG 28:0	511.4368	8.10E+05	8.73E+06	2.16E+06	2.88E+05	1.67E+05
DG 40:0	679.6246	2.54E+06	6.68E+06	1.25E+06	1.55E+05	1.78E+06
LPE 13:0	410.2313	2.14E+06	6.76E+06	7.16E+06	8.42E+06	4.38E+06
PE 28:0	634.4453	1.39E+07	3.24E+07	3.38E+07	3.66E+06	2.22E+07
PE 40:0	802.6331	8.21E+06	1.97E+07	1.97E+07	1.64E+06	9.07E+06
PG 28:0	665.4399	7.78E+06	3.33E+07	3.41E+07	4.89E+07	1.75E+07
PG 40:0	833.6277	7.66E+06	3.01E+07	3.06E+07	3.54E+07	1.16E+07
PI 34:0	837.5499	5.37E+06	1.70E+07	1.58E+07	1.40E+07	7.85E+06
PS 28:0	678.4352	9.11E+06	6.98E+07	5.31E+07	2.28E+07	7.38E+06
PS 40:0	846.6230	8.43E+06	5.23E+07	4.45E+07	2.06E+07	6.96E+06
HexCer 30:1;2	642.4950	1.76E+06	7.41E+05	9.00E+04	2.32E+04	2.31E+06
HexCer 35:1;2	712.5733	1.60E+06	5.90E+05	7.85E+04	1.66E+04	2.06E+06
Cer 32:1;2	508.4735	4.11E+06	3.00E+06	5.51E+05	2.44E+05	4.03E+06
Cer 35:1;2	550.5205	5.12E+06	4.09E+06	7.56E+05	3.17E+05	4.82E+06

Highest intensities could be found for PC, LPC, Cer, and HexCer internal standards while using LM1 or LM6, respectively (data not shown). This could be explained by the addition of ammonium formate, which favours the formation of formate adduct ions of these lipid species in negative ion mode. For other phospholipid classes and especially for free fatty acyls, the data in Table 3.37 showed a significant increase in intensity for LM3 a/b (0.005% MA). This in turn could be associated with the addition of methylamine (MA) since it supports deprotonation of these lipid classes. In this case, the addition of trimethylamine did not result in significant improvement compared to methylamine (see also Figure 3.11). In addition, several methylamine concentrations were tested using LM3 a/b (MeOH/$CHCl_3$ (5:1 v/v)): 0.005%, 0.01%, 0.05%, and 0.1%), showing highest intensities for 0.005% MA (data not shown). For this reason, 0.005% MA was added to all previously described solvent combinations for following experiments. LM6 which is used as standard solvent in our laboratory served as a reference for each test.

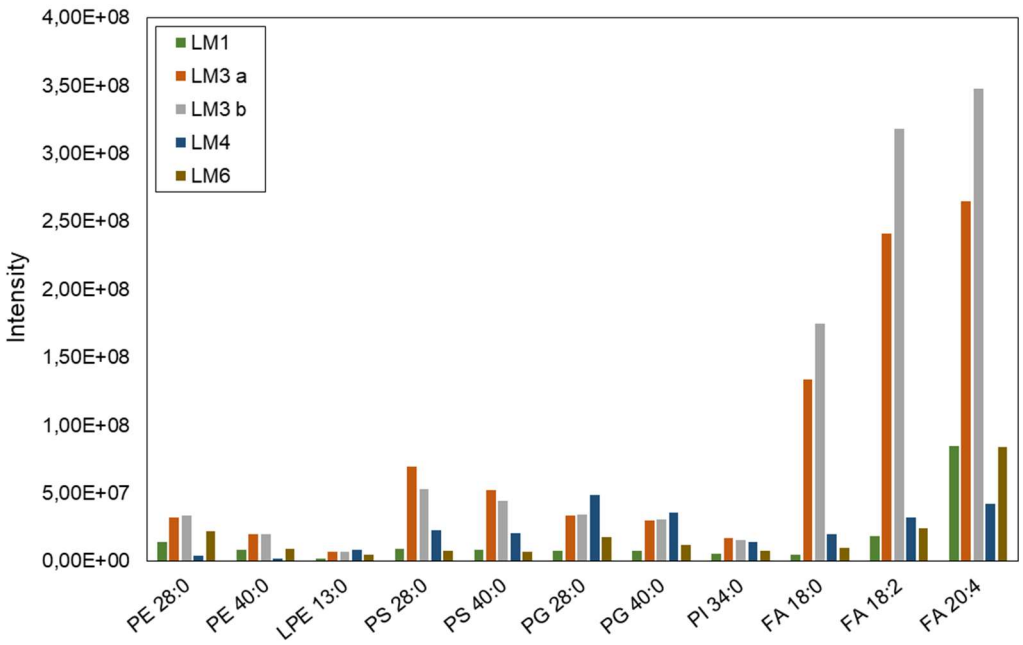


Figure 3.11: Bar charts of $[M-H]^-$ ions of *lyso*-phospholipid species, phospholipids, and FA species analyzed in negative ion mode are displayed. Samples were measured once each in different solvents, see Table 3.36. The corresponding adducts are listed in Table 3.37.

3.2.1 Evaluation of Lipid Species in Plasma

In order to evaluate the addition of bases, tests were carried out using plasma samples instead of fecal material. Numerous studies have already been published dealing with plasma, showing lipid species typically present in this sample material (181). For feces, only a few studies have been published to date (154) and it is still unclear, whether glycerophospholipids are present in sufficient amounts.

Three different aspects were considered regarding this experiment. First, different solvents were compared (LM2 a, LM3 a, LM5 a, LM7 a, and LM6, see Table 3.36). Second, a comparison between methylamines in each stock solution (H_2O and EtOH) was made, and finally the different methylamine concentrations in aqueous and ethanol-containing solutions were compared (0.005%, 0.01%, 0.5%, and 0.1%), see Table 3.38.

Data of this measurement reflected the results explained at the beginning. LM3 b (MeOH/$CHCl_3$ (5:1 v/v) with 0.005% methylamine) showed best results for lipid classes FA, PE, LPE, and PI, and therefore could be advantageously applied for the analysis of these lipid classes.

In a further experiment the stability of the two solvents LM3 b and LM6 was tested. For this purpose, the samples were measured and analyzed again after one week under

the same conditions. Data are shown in Figure 3.12. These data reflect the previous results. Lipid species of FA, PE, and PI were more stable in the methylamine-containing solvent.

For this reason, we would currently recommend dissolving the samples after extraction in both methylamine and formate containing solvents before measurement on the Q Exactive Orbitrap in order to obtain an optimal identification and quantification of all lipid classes.

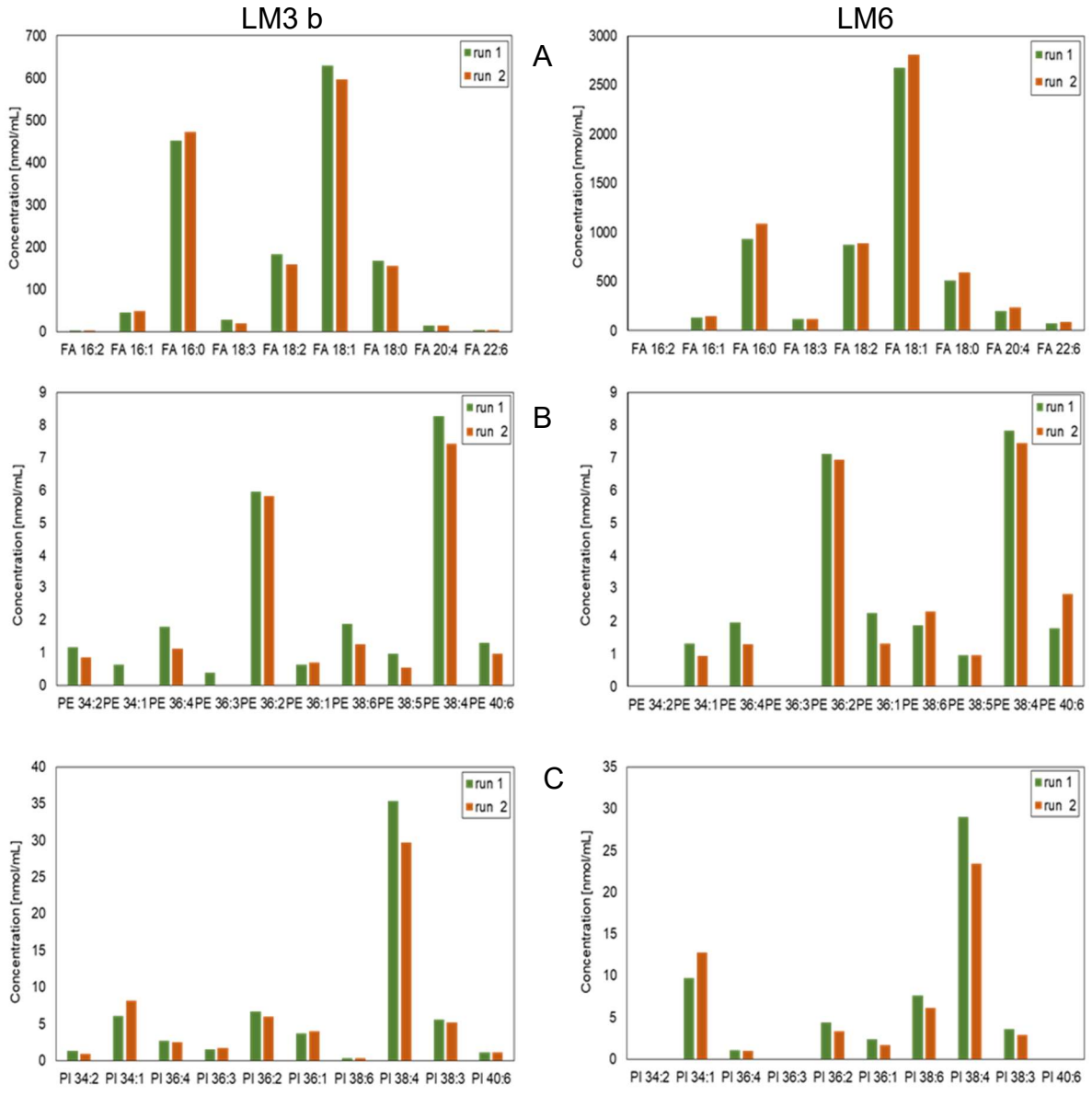


Figure 3.12: Panels show concentrations of A) $[M-H]^-$ ions of FA species, B) $[M-H]^-$ ions of PE species, and C) $[M-H]^-$ ions of PI species in a plasma sample containing either 0.005% methylamine (LM3 b, left) or formate (LM6, right). Samples were analyzed on two different days (run 1 and run 2) within one week.

Table 3.38: Intensities for FA, PE, and PI species in plasma samples obtained from different methylamine concentrations (0.005%, 0.01%, 0.05%, and 0.1%) in either LM3 a or LM3 b are displayed.

		Methylamine Concentration							
		LM3 a				LM3 b			
		0.005%	0.01%	0.05%	0.1%	0.005%	0.01%	0.05%	0.1%
FA	FA 16:2	2.91E+05	2.34E+05	2.58E+05	2.28E+05	3.29E+05	2.44E+05	2.42E+05	2.32E+05
	FA 16:1	1.28E+07	9.62E+06	1.08E+07	9.86E+06	1.33E+07	1.04E+07	1.07E+07	1.01E+07
	FA 16:0	1.32E+08	1.36E+08	1.30E+08	9.49E+07	1.29E+08	1.21E+08	1.14E+08	9.69E+07
	FA 18:3	7.63E+06	6.90E+06	5.51E+06	4.54E+06	9.55E+06	7.82E+06	4.80E+06	4.46E+06
	FA 18:2	4.90E+07	3.89E+07	3.97E+07	3.75E+07	5.75E+07	4.12E+07	3.92E+07	3.77E+07
	FA 18:1	1.70E+08	1.29E+08	1.47E+08	1.36E+08	1.90E+08	1.34E+08	1.44E+08	1.37E+08
	FA 18:0	5.11E+07	6.12E+07	5.04E+07	3.54E+07	4.47E+07	4.67E+07	4.33E+07	4.21E+07
	FA 20:4	3.68E+06	2.89E+06	3.58E+06	3.18E+06	3.92E+06	3.02E+06	3.41E+06	3.19E+06
	FA 22:6	1.00E+06	7.60E+05	9.24E+05	8.51E+05	1.07E+06	8.14E+05	9.11E+05	8.18E+05
PE	PE 34:2	4.30E+04	2.93E+04	2.06E+04	1.00E+04	3.12E+04	2.72E+04	2.50E+04	7.07E+03
	PE 34:1	3.16E+04	2.24E+04	1.84E+04	9.52E+03	2.39E+04	2.10E+04	2.33E+04	6.53E+03
	PE 36:4	8.87E+04	6.41E+04	5.93E+04	3.49E+04	9.95E+04	6.43E+04	5.83E+04	2.57E+04
	PE 36:3	1.44E+04	4.86E+03	5.89E+03	3.55E+03	1.58E+04	1.42E+04	1.18E+04	6.62E+03
	PE 36:2	2.39E+05	1.66E+05	1.56E+05	1.24E+05	2.26E+05	1.57E+05	1.68E+05	1.13E+05
	PE 36:1	5.25E+04	1.79E+04	2.41E+04	1.61E+04	3.90E+04	1.76E+04	3.55E+04	2.23E+04
	PE 38:6	8.58E+04	6.64E+04	7.46E+04	3.62E+04	9.56E+04	6.10E+04	6.51E+04	4.02E+04
	PE 38:5	7.53E+04	4.52E+04	3.53E+04	1.32E+04	4.99E+04	5.10E+04	3.77E+04	1.56E+04
	PE 38:4	3.15E+05	2.54E+05	2.12E+05	1.61E+05	3.19E+05	2.28E+05	2.35E+05	1.69E+05
	PE 40:6	7.95E+04	5.14E+04	4.92E+04	1.57E+04	7.00E+04	5.74E+04	5.64E+04	2.47E+04
PI	PI 34:2	1.71E+04	2.97E+03	3.55E+03	1.56E+04	3.37E+04	2.36E+04	2.94E+03	2.41E+04
	PI 34:1	1.43E+05	9.91E+04	8.99E+04	6.61E+04	1.32E+05	1.12E+05	1.28E+05	8.60E+04
	PI 36:4	5.59E+04	3.64E+04	3.32E+04	1.79E+04	6.16E+04	4.48E+04	3.75E+04	1.64E+04
	PI 36:3	2.70E+04	2.30E+04	3.26E+04	1.48E+04	3.43E+04	2.45E+04	3.34E+04	1.39E+04
	PI 36:2	1.56E+05	9.80E+04	7.51E+04	3.98E+04	1.57E+05	1.05E+05	9.19E+04	4.53E+04
	PI 36:1	8.11E+04	5.91E+04	5.23E+04	2.70E+04	9.01E+04	5.88E+04	5.95E+04	3.23E+04
	PI 38:6	6.22E+03	3.66E+03	7.74E+03	1.28E+04	5.51E+03	2.90E+03	1.03E+04	1.48E+04
	PI 38:4	8.04E+05	5.41E+05	4.41E+05	2.41E+05	8.36E+05	5.72E+05	5.12E+05	2.59E+05
	PI 38:3	1.18E+05	8.26E+04	6.10E+04	3.26E+04	1.29E+05	8.93E+04	7.62E+04	3.69E+04
	PI 40:6	2.73E+04	1.83E+04	1.58E+04	4.56E+03	2.84E+04	2.01E+04	1.76E+04	6.46E+03

3.2.2 Evaluation of Lipid Species in Feces

Finally, LM3 b and LM6 were compared for the identification and quantification of lipids contained in feces in negative ion mode. For this experiment, samples from three different voluntary donors, whose raw fecal materials were collected on four different days, were measured in triplicates. This experiment is independent of the intra-individual variance described in *3.1.4 Evaluation of Reproducibility Issues*. Figure 3.13 displays mass spectra from one human fecal sample analyzed in negative ion mode. Three different mass ranges used for analysing the two solvents (LM3 b and LM6) were compared. Spectra differed substantially between the solvents. While the intensities of the free fatty acyls were significantly higher in the methylamine-containing sample, internal standards of phospholipids were dominant in the mass range of *m/z* 500 – 960 in ammonium formate. Lysolipids could not be detected in either solvent. Mass range of phospholipid species showed, despite high intensity for the internal standards, no peaks that matched lipid species with sufficient intensities. Most species were detected at low intensities close or below the detection limit.

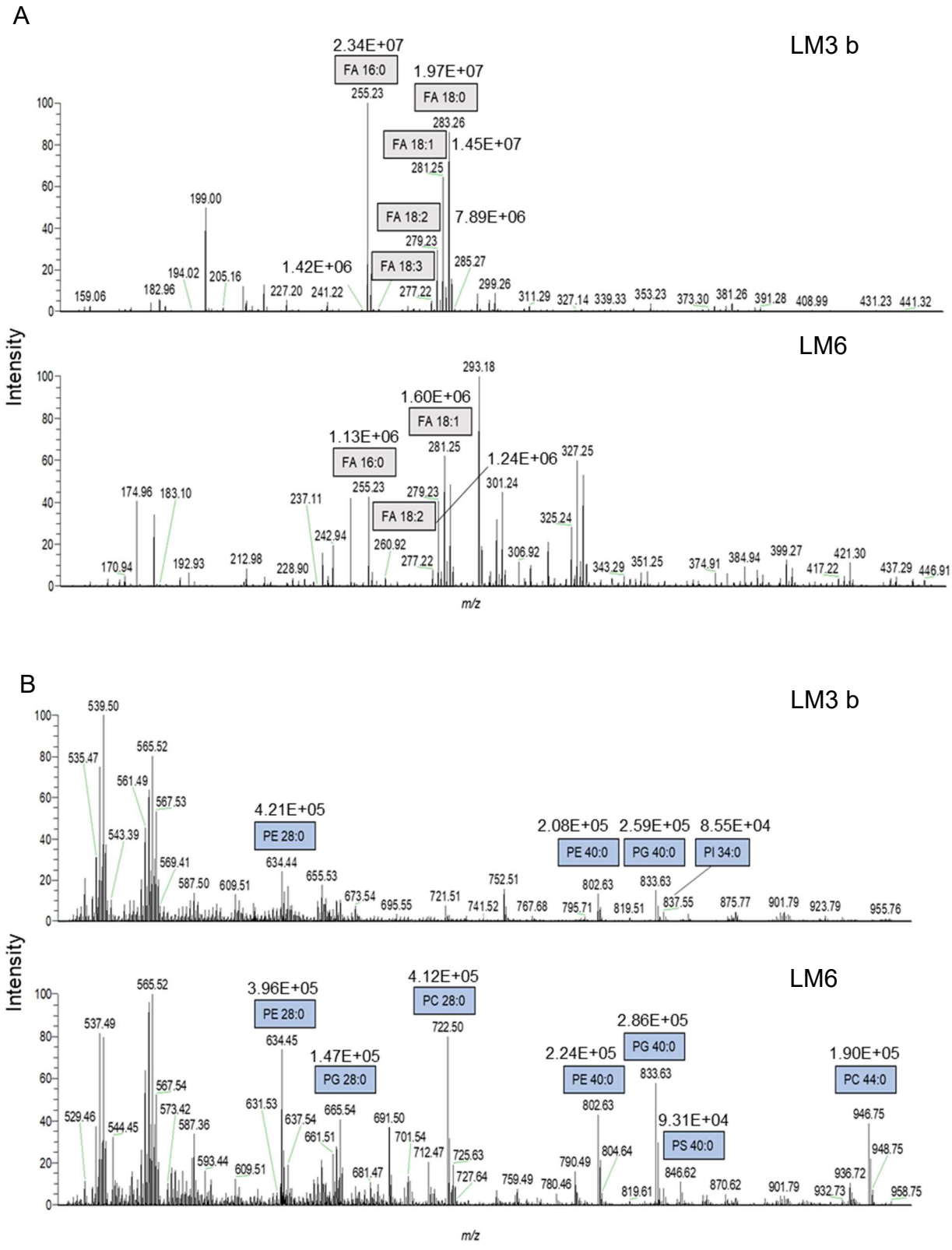


Figure 3.13: Mass spectra from one individual human fecal sample analyzed in negative ion mode are displayed. Panel A shows the mass range of FA species (*m/z* 150 – 450) and panel B of phospholipid species (*m/z* 500 – 960). The upper spectrum shows the sample with a 0.005 % methylamine containing solvent, whereas the lower spectrum displays the same sample dissolved in a formate containing solvent. Intensities of the detected species are shown. The corresponding adducts are listed in Table 3.37.

Table 3.39: Concentration of FA species analyzed with $[M-H]^-$ ions from three different donors, whose raw fecal materials were collected on four different days are displayed. Samples were dissolved in MeOH/$CHCl_3$ (5:1 v/v) containing 0.005% methylamine in ethanol (LM3 b). Each sample was analyzed in triplicates.

	FA Sum	
	Mean (n=3) [nmol/mg dw]	CV [%]
donor A1	**426.41**	5.4
donor A2	**408.30**	7.6
donor A3	**381.71**	3.5
donor A4	**377.54**	4.2
donor B1	**430.33**	3.0
donor B2	**401.82**	6.2
donor B3	**411.60**	2.6
donor B4	**351.44**	4.0
donor C1	**444.69**	1.2
donor C2	**405.94**	4.7
donor C3	**401.05**	4.9
donor C4	**398.74**	4.4

Table 3.39 shows that analysis of free fatty acyls in methylamine containing solvent LM3 b revealed a wide range of species. Most common species were listed in Table 3.40 and in the corresponding species profile in % of total FA in Table 3.41. Concentrations of free fatty acids were corrected for background.

Since these species were also detected at high intensities in the background and all samples showed almost an identical profile, these data should be validated similarly as shown for DG and TG.

Table 3.40: Calculation of CV for free fatty acyl species from three different donors, whose raw fecal materials were collected on four different days. Samples were dissolved in MeOH/$CHCl_3$ (5:1 v/v) containing 0.005% methylamine in ethanol (LM3 b). Each sample was analyzed in triplicates. Evaluation of $[M-H]^-$ ions of FA species. Data were corrected for the background detected in the internal standard blanks.

	FA 16:1		FA 16:0		FA 18:3		FA 18:2		FA 18:1		FA 18:0		FA 20:1		FA 20:0	
sample	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]	Mean (n=3) [nmol/ mg dw]	CV [%]
donor A1	**1.11**	5.8	**121.70**	5.5	**6.22**	4.9	**37.51**	4.6	**80.91**	6.0	**104.66**	6.1	**1.63**	4.1	**2.96**	6.5
donor A2	**1.10**	7.5	**115.52**	7.8	**6.66**	7.9	**37.20**	8.2	**74.76**	8.1	**100.69**	8.0	**1.52**	8.3	**2.82**	7.0
donor A3	**1.03**	3.6	**106.92**	3.6	**6.55**	3.8	**36.47**	3.5	**69.19**	3.6	**91.13**	4.1	**1.34**	4.0	**2.54**	2.7
donor A4	**1.03**	3.3	**105.10**	3.8	**6.71**	4.1	**37.28**	4.4	**67.98**	4.8	**90.15**	4.7	**1.28**	5.5	**2.48**	4.8
donor B1	**1.14**	3.2	**125.42**	3.0	**6.78**	3.3	**37.56**	2.9	**78.14**	3.1	**105.56**	3.6	**1.61**	3.4	**3.10**	4.5
donor B2	**1.04**	5.6	**114.52**	7.8	**7.14**	9.5	**36.32**	5.0	**72.91**	5.3	**98.77**	4.8	**1.47**	5.7	**2.86**	6.6
donor B3	**1.09**	2.8	**117.39**	2.4	**8.34**	4.0	**37.96**	3.1	**74.68**	3.1	**98.77**	2.9	**1.43**	1.8	**2.93**	0.9
donor B4	**0.93**	4.1	**95.26**	3.8	**7.79**	4.5	**33.99**	4.4	**63.00**	4.3	**85.05**	4.2	**1.18**	4.1	**2.33**	3.4
donor C1	**1.17**	0.6	**129.08**	1.8	**7.41**	4.9	**39.49**	0.7	**82.54**	1.8	**108.94**	0.9	**1.68**	0.4	**3.18**	1.5
donor C2	**1.06**	5.7	**114.68**	5.5	**7.75**	3.2	**37.97**	5.4	**75.84**	3.3	**97.22**	4.5	**1.49**	5.1	**2.83**	6.0
donor C3	**1.05**	4.7	**113.94**	5.0	**8.37**	4.0	**38.33**	4.1	**73.73**	5.0	**94.76**	5.6	**1.42**	5.7	**2.78**	6.0
donor C4	**1.04**	5.8	**113.22**	5.7	**8.40**	4.1	**38.01**	5.6	**72.55**	3.1	**95.03**	3.4	**1.40**	3.8	**2.87**	6.1

Table 3.41: Species profile in % total FA of the data listed in Table 3.40.

Species	donor A1 [%]	donor A2 [%]	donor A3 [%]	donor A4 [%]	donor B1 [%]	donor B2 [%]	donor B3 [%]	donor B4 [%]	donor C1 [%]	donor C2 [%]	donor C3 [%]	donor C4 [%]
FA 16:1	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
FA 16:0	34.5	34.4	34.4	34.2	35.3	34.6	34.7	33.5	34.9	34.3	34.5	34.5
FA 18:3	1.7	1.9	2.0	2.1	1.9	2.1	2.4	2.6	2.0	2.2	2.4	2.5
FA 18:2	10.3	10.7	11.3	11.7	10.3	10.6	10.9	11.4	10.4	11.0	11.2	11.2
FA 18:1	22.3	21.6	21.5	21.4	21.4	21.4	21.4	21.3	21.7	22.0	21.7	21.4
FA 18:0	29.4	29.7	29.0	29.0	29.5	29.6	28.9	29.5	29.3	28.8	28.5	28.7
FA 20:1	0.5	0.5	0.4	0.4	0.5	0.4	0.4	0.4	0.5	0.4	0.4	0.4
FA 20:0	0.9	0.9	0.9	0.9	1.0	1.0	1.0	0.9	0.9	0.9	0.9	1.0

3.2.3 Summary

These data showed that a direct measurement of (*lyso*)glycerophospholipids is not possible for feces despite enhanced intensities in negative ion mode upon MA addition. For other samples, such as plasma or tissue, methylamine provided higher intensities for lipid classes which are preferably deprotonated in negative ion mode. Therefore, it could be useful to analyze samples with both methylamine and formate containing solvents in order to cover more lipid classes.

Currently, there are hardly any studies regarding the human fecal lipidome. The literature in this respect is not uniform and a quantification of phospholipids is not sufficiently described either (154, 155, 182). According to a study published in 1970 by Erb et al. (183), the daily fecal lipid extraction was found between 0.55 and 1.93 g/day. The largest proportion was accounted by esterified and free neutral sterols, 25% was free fatty acyls, whereas phospholipids could only be detected in small amounts.

Analysis of free fatty acyls in methylamine provided good signals, but data should be interpreted with caution due to high amounts of FA in the background. It seems that there are only small amounts of other lipid classes, especially phospholipids, present in feces, hence a direct measurement is not possible. “Shotgun” methods seem to be too insensitive. Therefore, chromatographic techniques, such as LC-MS, should be used to increase the sensitivity for polar lipids (184-186).

Another possibility to detect phospholipids might be to separate polar from non-polar lipids, as described for example by Vale et al. (187). The three-phase lipid extraction (3PLE) technique uses a single step liquid-liquid extraction and allows both extraction and fractionation of lipids by their polarity as it consists of an aqueous and two organic phases. The neutral lipids such as DG and TG are primarily extracted in the more non-polar upper organic phase, whereas the middle organic phase contains mainly glycerophospholipids. Thus, suppression of TG species could be reduced.

The reason for low phospholipid concentrations might be that bacterial lipids are not accessible due to insufficient homogenization of the fecal material. As already described in detail for DG and TG species, a sufficient homogenization of this sample material is challenging. Phospholipids could perhaps be made accessible through an additional homogenization step, e.g. by using the Precellys® homogenizer or by adding a detergent. Another reason may be their low concentration in feces. To answer this question, further tests are needed.

4. Conclusion

This thesis describes the development of a mass spectrometric method for the identification and quantification of lipid species in human fecal material using flow injection analysis (FIA) coupled to a high-resolution FTMS instrument. This is, to our knowledge, the first method using a conventional LC pumping system to infuse crude lipid extracts and to analyze DG and TG in human feces. Up to now, only a few studies on the fecal lipidome exist which is most likely related to the difficulties faced with this sample material (134, 154).

The proposed method has a short run time of four minutes per sample, including MS2 measurements, facilitating a high sample throughput necessary for clinical studies. Validation of the novel method demonstrated its suitability for large scale studies despite the higher variations observed for some samples. These variations are related to inhomogeneity of samples and lipolytic activity that requires further investigations considering pre-analytical issues as an essential part of lipidomic workflows and their standardization (165, 166, 188). Therefore, we recommend performing measurements in triplicates when high accuracy is needed. In this regard, sampling is very important since metabolites are distributed in a highly heterogeneous way in feces and homogenization of larger quantities is recommended (189). For determination of the concentrations of DG and TG species, native samples are preferred due to better stability over several days at RT. Species profiles showed stable values and could therefore be used for studies to evaluate a more precise statement about DG and TG species.

Evaluation of the data also showed that using direct injection mass spectrometry is not suitable for identification and quantification of other lipid classes besides DG and TG. Despite optimization of the negative ion mode by addition of a base to enhance signals of phospholipids, this method could not detect substantial amounts of phospholipids in fecal samples.
For this reason, the sensitivity of the method should be increased for subsequent experiments in order to be able to detect polar lipids, for example by using a LC-MS in hydrophilic interaction liquid chromatography (HILIC) mode.

In summary, the presented method provides a valuable tool to quantify DG and TG species as major lipid classes in human fecal samples. These data could be a first step to unravel the fecal lipidome and get more insight into its role for health and disease.

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VIII Selbstständigkeitserklärung

“Ich, Ertl, Verena Magdalena; geb. Lichtinger geboren am 27.12.1990 in Mallersdorf-Pfaffenberg, erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

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